Investigation of the Mechanism of Action of Ingenol Mebutate and Identification of Potential Key Players in Keratinocyte Growth Regulation

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Sandra Nicole Freiberger

aus

Deutschland

Promotionskomitee

Prof. Dr. Reinhard Dummer (Vorsitz)

Prof. Dr. Günther Hofbauer (Leitung der Dissertation)

Prof. Dr. Onur Boyman

Prof. Dr. Gian-Paolo Dotto

Prof. Dr. Ian Frew

Zürich, 2015

Table of Content

I. Zusammenfassung

Das Plattenepithelkarzinom (SCC) der Haut ist der zweithäufigste Hautkrebs des Menschen nach dem Basalzellkarzinom (BCC). Seine Inzidenz ist über die vergangenen Dekaden kontinuierlich angestiegen. Des Weiteren ist das Plattenepithelkarzinom nach dem Melanom die zweithäufigste Ursache von hautkrebs-assoziierten Todesfällen, da im Unterschied zum häufigeren BCC, SCCs mit fortschreitender Tumordicke zunehmend Metastasen bilden. Eine frühe intraepitheliale Vorstufe bei SCC ist die aktinische Keratose (AK), welche sich auf den sonnengeschädigten Arealen der Haut, wie Gesicht, Kopfhaut und Unterarmen, bildet. Die Läsionen werden durch chronische UV-Exposition induziert, welche Mutationen in der DNA von Keratinozyten verursacht. Wenn Mutationen auftreten, werden sie normalerweise von spezifischen Proteinen der DNA-Reparaturmaschinerie innerhalb der Zelle erkannt, wobei der Zellzyklus angehalten wird, bis die DNA repariert wurde. Jedoch werden manche Mutationen nicht korrekt repariert und bestehen somit weiterhin. Solche Mutationen resultieren in einer Aktivierung oder in erhöhter Expression von Onkogenen oder in Inaktivierung von Tumorsuppressorgenen, was wiederum zu unkontrollierter Zellproliferation und Tumorentstehung führt. Zusätzlich gibt es Mutations-unabhängige Prozesse, welche eine veränderte Proteinexpression, z.B. von bestimmten Memranrezeptoren zur Folge haben, und damit zu Tumorwachstum führen.

Organtransplantierte (OTR) haben ein 250-fach erhöhtes Risiko, AK zu entwickeln und ein 100-fach erhöhtes Risiko, SCC zu bekommen. Grund dafür ist die Einnahme von bestimmten Immunsuppressiva, die zum Prozess der Entstehung beitragen, indem sie die Tumorabwehr hemmen und eine direkte Einwirkung auf Keratinozyten haben. Da sich ein kleiner Teil von AKs zu invasiven SCCs weiterentwickelt, und AKs narbenfrei entfernbar sind, strebt man in der klinischen Praxis eine Behandlung von AK an. Mehrere Optionen stehen für die Behandlung von AK zur Verfügung. Jedoch dauern viele dieser Behandlungen Wochen bis Monate und sind somit sehr zeitaufwendig. Nebenwirkungen wie lokale Entzündungen und Beschwerden sind die Regel und dauern entsprechend der Behandlungsdauer an. Kürzlich wurde eine neue Substanz, Ingenol Mebutate (IM), zur Behandlung von AK in den USA und Europa zugelassen, welches den Vorteil einer sehr kurzen Anwendungsdauer ohne systemische Wirkung bietet. IM wird

in der Klinik routinemässig eingesetzt und ist sehr effektiv, während sein Wirkmechanismus noch nichtaufgeklärt ist.

Das Hauptziel der vorliegenden Doktorarbeit war es, den Wirkmechanismus von IM auf Keratinozyten, dem primären Ziel der Behandlung, zu untersuchen. Dazu haben wir zunächst den direkten Effekt von IM auf Lebensfähigkeit und Proliferation von gesunden und kanzerösen Keratinozyten und Zelllinien untersucht. Des Weiteren analysierten wir die Genexpressionsprofile von IM-behandelten gesunden Keratinozyten und Plattenepithelkarzinomzellen von Patienten und definierten die involvierten Signalwege. Hier fanden wir, dass die Wirkung von IM PKCδ-abhängig ist und über den MEK/ERK-Signalweg vermittelt wird. Zudem identifizierten wir die Interleukinrezeptoren IL1R2 und IL13RA2 als neue Zielgene von IM. Beide Gene zeigten eine deutliche Aufregulation nachdem die Zellen mit IM behandelt wurden. Des Weiteren konnten wir in funktionalen Experimenten die Wichtigkeit dieser Zielgene für die Vitalität der Zellen nach IM-Behandlung zeigen.

Wie oben erwähnt kann die veränderte Expression von verschiedenen Proteinen zur Entwicklung von AK und SCC beitragen. Weitere in dieser Dissertation beinhaltete Forschungsprojekte beschäftigen sich mit der Rolle der Membranrezeptoren RAGE, TLR4 und CD32 bei der Entstehung und dem Voranschreiten des Plattenepithelkarzinoms. Einerseits konnten wir zeigen, dass die Stimulation des Rezeptors RAGE mit einem seiner Liganden, S100A8/A9, zu erhöhter Keratinozytenproliferation führt. Andererseits fanden wir eine Rolle in der negativen Regulation der Proliferation für Toll-like Rezeptor 4 (TLR4), dessen Überexpression zu verminderter Proliferation führt, während eine Herunterregulation die Proliferation der Zellen erhöht. Zusätzlich identifizierten wir Fcγ Receptor IIB (CD32B) als negativen Regulator der Proliferation und fanden diesen Rezeptor hoch exprimiert auf differenzierten Zellen. Ähnlich wie bei TLR4, führt eine erhöhte CD32B-Expression zu verminderter Proliferation, während eine verminderte Expression des Rezeptors die Proliferation erhöht. Somit vermuten wir eine Tumorsuppressor-Funktion dieses Rezeptors.

Wie zuvor erwähnt, haben OTR auf Grund von immunsupprimierenden Medikamenten ein erhöhtes Risiko, AK und SCC auf Sonnen-exponierter Haut zu entwickeln. In einem unserer Projekte haben wir untersucht, wie das immunsupprimierende Medikament Cyclosporin A (CsA) in Kombination mit UVA-Licht einen Anstieg des Transkriptionsfaktors ATF3 fördert und dabei zur Tumorentwicklung beiträgt.

Zusammengefasst liefern die Ergebnisse der vorliegenden Dissertation ein besseres Verständnis des Wirkmechanismus von IM. Diese Resultate helfen dabei, topische Medikamente auf Ingenol-Basis zu verbessern und zeigen wichtige Signalwege und neue Zielgene auf, die als Ansatzpunkt für neue therapeutische Herangehensweisen genutzt werden können. Die Entdeckung, dass verschiedene Membranrezeptoren an der Entstehung des Plattenepithelkarzinoms beteiligt sind, liefert neue Einsichten in die Komplexität der Erkrankung und ebnet den Weg für neue, gezielte Behandlungsmöglichkeiten. Die in einer unserer weiteren Arbeiten gezeigte Potenzierung des Transkriptionsfaktors ATF3 durch die Kombination von CsA und UVA-Licht trägt dazu bei, den Mechanismus zu verstehen, durch den immunsupprimierte Patienten Plattenepithelkarzinome entwickeln und erwägt eine aufkommende Rolle von UVA in der Tumorentstehung.

II. Summary

Squamous cell carcinoma (SCC) is the second most common human skin cancer after basal cell carcinoma (BCC). Its incidence has continuously risen over the last decades. Moreover, SCC is the second-leading cause of skin cancer-related deaths behind melanoma. While BCC hardly metastasizes, SCC does so with increasing tumor thickness and loss of differentiation. The early intraepithelial lesion of SCC is actinic keratosis (AK), developing on sun-exposed surfaces of the skin, like lower face, scalp and lower arms. These lesions are induced by chronic UV exposure mutating DNA of keratinocytes. When mutations occur, they are generally sensed by the DNA repair machinery. The cell cycle gets arrested until the DNA has been repaired. However, some mutations do not get repaired correctly, so that the mutations persist. Such mutations result in an activation or increased expression of oncogenes or inactivation of tumor suppressor genes, leading to uncontrolled cellular proliferation and tumor formation. Additionally, mutation-independent processes lead to differentially expressed proteins which in turn may play a role in tumor development and progression, among them certain membrane receptors.

As a group with increased risk for SCC, organ transplant recipients (OTR) have a 250-fold higher risk to develop AK and a 100-fold higher risk to develop SCC because several immunosuppressive drugs, e.g. azathioprine or cyclosporine A (CsA), allow and contribute to the process of tumor formation. A minority of AK progress to invasive SCCs. At the intraepithelial stage of AK, treatment is highly effective and usually non-surgical. Several treatment modalities are used for the treatment of AK. However, most treatments take weeks to months and are therefore very time-consuming. Furthermore, these treatments are accompanied by side-effects like inflammation and tenderness during the whole period of treatment. Recently, a new compound, ingenol mebutate (IM), has been registered in USA and Europe for the treatment of AK, characterized by a very short treatment duration without systemic effects. Although IM is now routinely used in the clinic and found to be highly effective, the exact mechanism of action has not been elucidated.

The main aim of this PhD thesis was to investigate the mechanism of action of IM on keratinocytes, the main target of the treatment. Therefore, we first studied the direct effect of IM on viability and proliferation of healthy and cancerous primary

keratinocytes and cell lines. We furthermore analyzed the gene expression profile of treated keratinocytes and patient cells and determined involved signaling pathways. Here, we found the effect of IM to be mediated by PKCδ-dependent MEK/ERK signaling. Moreover we were able to identify IL1R2 and IL13RA2 as novel target genes of IM. Both genes showed a prominent upregulation after IM treatment in keratinocytes and SCC cells. Furthermore, we showed in functional experiments the importance of these target genes for the viability of IM-treated cells.

As described above, differential expression of various proteins can contribute to AK and SCC development. Further projects included in this thesis had the aim to elucidate the role of the membrane-bound receptors RAGE, TLR4 and CD32B in these processes. On the one hand, we showed that stimulation of the receptor RAGE with one of its ligands, S100A8/A9, led to enhanced keratinocyte proliferation. On the other hand, we found a role in negative regulation of proliferation for Toll-like receptor 4 (TLR4), whose overexpression led to decreased proliferation, while a knockdown of increased the cell proliferation. Additionally, we found Fcγ receptor IIB (CD32B) to be a negative regulator of proliferation and to be highly expressed in differentiated cells. Similar to TLR4, an elevated expression of CD32B led to decreased proliferation, while a knockdown of the receptor highly increased the proliferation. Therefore, we suggest it to have a tumor-suppressing function.

As mentioned above, OTR have an increased risk to develop AK and SCC on sun-exposed surfaces of the skin due to the effect of immunosuppressive drugs. In one project, we investigated how the above-mentioned immunosuppressive drug CsA in combination with UVA light can promote an increase of the transcription factor ATF3, thereby enhancing tumor development.

In summary, the results of the current thesis provide a better understanding of the mechanism of action of IM. These results will help to improve ingenol-based topical treatments and point out important pathways and new target genes that could serve as starting point for new or potentially improved therapeutic approaches. The discovery of several membrane receptors being involved in the development of SCC gives new insights in the complexity of the disease and proposes new therapeutic targets for

cancer therapy. Our work showing a potentiated effect of CsA and UVA on the transcription factor ATF3 contributes to further understanding of the mechanisms by which immunosuppressed patients develop SCC and suggests an emerging role for UVA in skin tumorigenesis.

III. Introduction

1. Skin overview

1.1. Structure

The human skin is the largest human organ with a total area of 1.6-2 m^2 in adults and is divided into the epidermis and the dermis. Underneath the dermis is the subcutaneous tissue, the hypodermis, located which contains mainly fatty tissue and collagen (Fig. 1) (Rassner, 2007). Epidermis and dermis are separated by the basal membrane. Depending on the body site, the epidermis is between 0.05 mm and 1.5 mm thick, while the thickness of the dermis ranges between 1.5 mm and 4.0 mm (National Cancer Institute, 2014b).

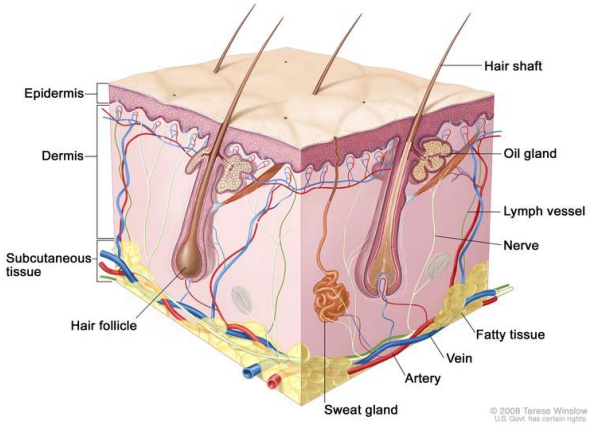


Fig. 1: Layers of the human skin (National Cancer Institute)

The epidermis consists of several layers. The outermost one is the *stratum corneum*, followed by *stratum granulosum*, *stratum spinosum* and *stratum basale* (Fig. 2). The main components of the epidermal layers are keratinocytes (95% of the cells) in various differentiation states depending on the layer (Simpson et al., 2011). Besides keratinocytes, there are few other cell types located in the epidermis: melanocytes, Langerhans cells and Merkel cells. The cellular dermis is built by fibroblasts, mast cells and histiocytes (McGrath, 2004).

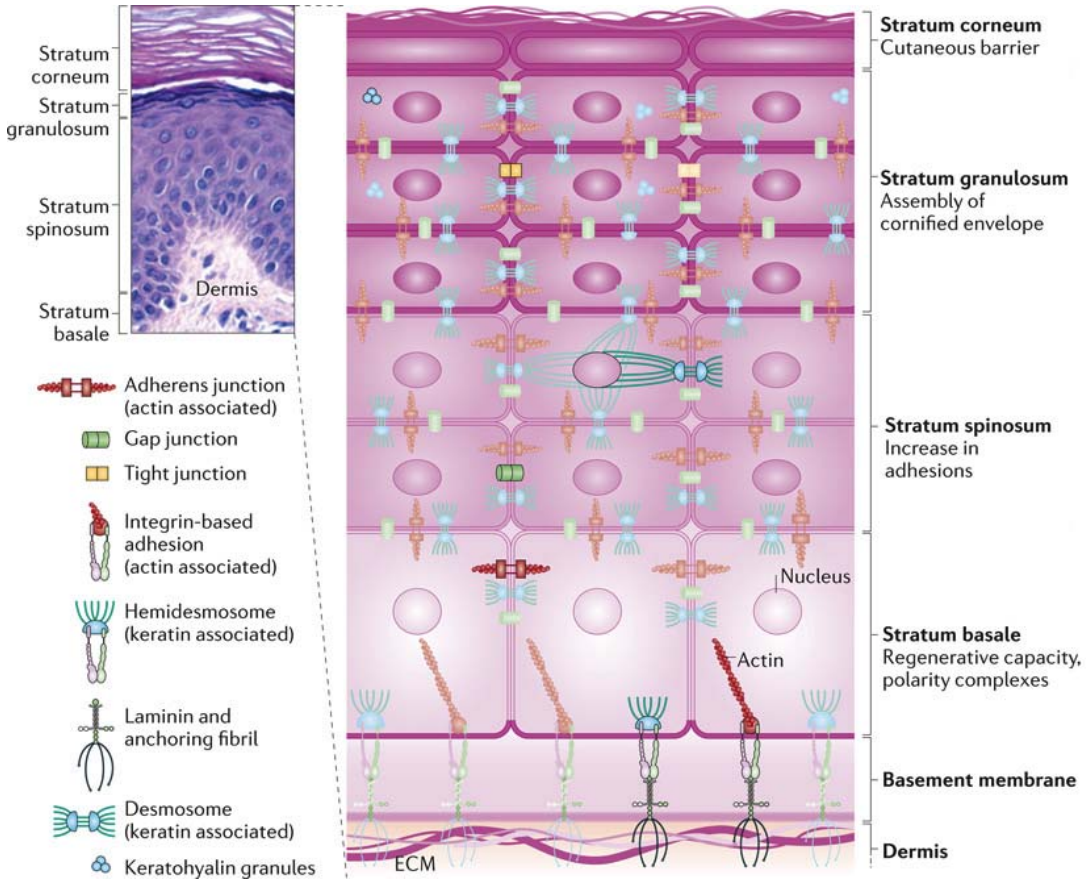


Fig. 2: Structure of the human epidermis (modified) (Simpson et al., 2011).

1.2. Cell types

1.2.1. Keratinocytes

Keratinocytes originate from a small population of stem cells, located in the hair follicle (Hsu et al., 2011). During their differentiation process, keratinocytes move through the epidermal layers until they reach terminal differentiation in the *stratum corneum*, where they die and detach. Therefore, a balance between newly generated keratinocytes in the hair follicle and dying keratinocytes in the top layer is important (McGrath, 2004). The turnover of healthy epidermis was estimated to be 28 days, while in pathological conditions this turnover rate is altered. For example, the turnover of psoriatic epidermis shrinks to four days on average (Rothberg et al., 1961).

Every step in keratinocyte differentiation is characterized by the expression of specific differentiation markers. Basal keratinocytes express keratin 5 and keratin 14. Keratinocytes of the spinous layer are more differentiated than basal keratinocytes and express keratin 1 and keratin 10. Cells of the granular layer show a decrease in keratin

1 and keratin 10. Furthermore, markers for terminal keratinocyte differentiation are induced. These proteins are involucrin, which is involved in the early formation of the cornified envelope, loricrin, the main component of the cornified envelope, and filaggrin (Kypriotou et al., 2012). A fault in the process of keratinocyte differentiation can lead to cancer development.

1.2.2. Melanocytes

Melanocytes are derived from the neural crest (Regad, 2013) and are found in the *stratum basale*. These cells synthesize melanin, a UV-absorbing pigment protecting the DNA of keratinocytes from damage (Hirobe et al., 2002). Melanocytes can give rise to benign melanocytic tumors like nevi or to its malignant counterpart melanoma, causing most skin cancer deaths (American Cancer Society, 2014-08-11).

1.2.3. Merkel cells

Merkel cells are located in the *stratum basale* of the epidermis. They are connected to nerve endings and are thought to function as mechano-receptors. Furthermore, Merkel cells are the origin of Merkel cell carcinoma, a relatively rare but aggressive skin tumor (Halata et al., 2003).

1.2.4. Fibroblasts

Fibroblasts are the main cellular component of the dermis. They produce several features of the extracellular matrix (ECM) like fibronectin and collagen. Besides maintaining the homeostasis of the ECM, fibroblasts play a crucial role in wound healing and interact with keratinocytes at the basal membrane (Kalluri and Zeisberg, 2006; Sorrell and Caplan, 2004). Dermal fibroblasts probably also contribute to SCC formation in sun-damaged skin (Hu et al., 2012).

1.2.5. Immune cells

Besides keratinocytes, which also possess certain immunologic activity, there are other members of the immune system present in the skin. IN the epidermal compartment, there are antigen-presenting Langerhans cells, a subgroup of dendritic cells. These cells stimulate T cell-dependent immune responses. Neutrophils can be recruited to the epidermis when inflammation occurs. Epidermal resident T cells may be joined by T cells recruited after initiation of an immune response (Nestle et al., 2009; Salmon et al.,

1994). The dermis contains dendritic cells, macrophages, natural killer cells, regulatory T cells, γδ T cells and $CD4^+$ or $CD8^+$ T cells (Nestle et al., 2009).

2. Cancer overview

2.1. Definition

The American Cancer Society defines cancer as a group of more than 100 diseases, characterized by the uncontrolled growth of abnormal cells (American Cancer Society, 2014-08-11). IN contrast to healthy cells, cancer cells acquire certain abilities allowing them to survive, grow and spread. Each cancer type possesses some or all of these abilities summarized as the hallmarks of cancer by Hanahan and Weinberg (Hanahan and Weinberg, 2000).

2.2. Hallmarks of cancer

In the year 2000, Hanahan and Weinberg defined the hallmarks of cancer, summarizing abilities that were adapted by cells leading to their uncontrolled spreading and growth. They described six capabilities, being "self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis" (Hanahan and Weinberg, 2000), all of which participate in the development and progression of cancer. Great efforts in cancer research over the years have led to new insights causing Hanahan and Weinberg to update the hallmarks of cancer and include the ability to evade the immune system and the ability to modify cellular metabolism to guarantee energy supply for the enhanced cancer cell proliferation (Hanahan and Weinberg, 2011). Furthermore, so-called "enabling characteristics" were added which are genome instability and mutations, and tumor-promoting inflammation, both of them supporting the development of the hallmarks. Recent progress in molecular biology, pharmacy and medicine led to the development of new therapeutic strategies that target these cancer-specific hallmarks whereas the cancer in turn may acquire certain resistance mechanisms to evade (Hanahan and Weinberg, 2011), making ongoing research indispensable.

3. Skin Cancer

3.1. Melanocytic and non-melanocytic skin cancer

Skin cancer can be divided into two subclasses, melanocytic and non-melanocytic skin cancer, each of them arising from different cells of origin. Melanoma derives from melanocytes, pigmented cells located in the basal layer of the epidermis (Regad, 2013). Statistics of the National Cancer Institute of the NIH estimate 76.100 new cases of melanoma in the US for 2014 representing 4.6% of all new cancer cases. The estimated number of deaths from melanoma is estimated at 9.710 representing 1.7% of all cancer deaths. The 5-year survival rate of patients with cutaneous melanoma without metastasis reaches 98.1%, whereas patients with regional or distant metastases have a greatly impaired survival of 62.6% and 16.1%, respectively (National Cancer Institute, 2014a).

New estimates from the National Cancer institute claim more than 2 million new cases of non-melanocytic skin cancer for 2014 in the US, resulting in up to 1000 deaths (National Cancer Institute, 2014c). The two major groups of non-melanoma skin cancer are basal cell carcinoma (BCC) with an incidence of 407/100.000 men and 212/100.000 woman yearly (Miller and Weinstock, 1994) and squamous cell carcinoma (SCC) with an incidence of 12 / 100.00 women and 19.1 / 100.000 men (Birch-Johansen et al., 2010). As reflected by the name, BCC was originally thought to derive from keratinocytes located in the basal layer of the epidermis. However, newer finding consider the stem cells in the hair follicle as cell of origin in BCC (Grachtchouk et al., 2011; Schirren et al., 1997).

SCC was commonly thought to develop from squamous keratinocytes located in the outer part of the epidermis, but recent reports suggest that SCC can arise from different sources, like hair follicle stem cells but also from interfollicular stem cells (Lapouge et al., 2011; White et al., 2011). Apart from that, there are several other non-melanocytic, rare skin cancers, e.g. Merkel cell carcinoma, Kaposi's sarcoma, cutaneous T-cell lymphoma or sarcoma (Cassarino, 2012).

3.2. Actinic keratosis (AK) and cutaneous squamous cell carcinoma (SCC)

Actinic keratoses (AK) are hyperkeratotic macules or plaques arising on sun-exposed surfaces such as face, scalp or lower arms. AKs develop from atypically proliferating keratinocytes and are considered as precancerous lesions or squamous cell carcinoma

(SCC) *in situ* (Ackerman and Mones, 2006). The grading of AK involves different stages of keratinocyte intraepidermal neoplasia (KIN), whereas KIN I is limited to the lower third of the epidermis including the basal layer, KIN II affects the lower two thirds of the epidermis and KIN III affects all of the epidermis. However, new studies showed that AK does not necessarily traverse through all three stages to become invasive SCC but that SCC may emerge from KIN I lesions in the basal keratinocyte layer (Fernandez-Figueras et al., 2014). Additionally, clinical studies revealed that about 8% of all AK progress to invasive SCC over a period of 10 years from the first AK diagnosis (Glogau, 2000).

The incidence of AK and SCC is rapidly increasing and new estimates from the US showed that about 2% of the patients actually die from cutaneous SCC (Karia et al., 2013). AK and SCC mainly occur in the elderly Caucasian population with a higher incidence in areas with high sun exposure such as Australia (Frost et al., 2000). The major risk factor for the development of AK and SCC is chronic exposure to ultra violet (UV) light, associated with sunburns and fair skin. Moreover, genetic disorders, e.g. xeroderma pigmentosum, bear a high risk (Euroderm, 2011). Organ transplant recipients (OTR) under chronic immunosuppression have a 100-fold increased risk to develop SCC compared to the general population, with 40% of the immunosuppressed AK patients developing invasive SCC (Stockfleth et al., 2002). While BCC rarely metastasize (0.0028 - 0.05% of all cases) (Rubin et al., 2005), SCC have a metastatic potential, reported to be between 0.1% and 9.9% of the cases (Weinberg et al., 2007). Generally, the prognosis of SCC depends on differentiation, invasion depth and location of the tumor. Poor differentiation and deep invasion correlate with a worse prognosis. Regardless of differentiation, tumor location plays an important role whereas ear and lip associate with worse outcome (Cassarino, 2012). As we fail to understand to which AK will progress to invasive SCC, treatment of AK is generally recommended and practiced.

3.2.1. Molecular pathogenesis

The risk factors mentioned in the previous section affect the skin on a molecular basis which will be discussed in this section in more detail.

3.2.1.1. Role of UV light

Chronic sun exposure or, more specifically, chronic low-level exposure to UV light is the most common cause of AK and SCC. UV light can be distinguished by wavelength into

UVA (320–400 nm), UVB (290–320 nm) and UVC (< 290 nm). UVC has the highest energy, but most of it gets absorbed in the atmosphere before reaching the epidermal surface. UVB and UVA penetrate deeper into the epidermis and dermis (Fig. 3) (Lee et al., 2013).

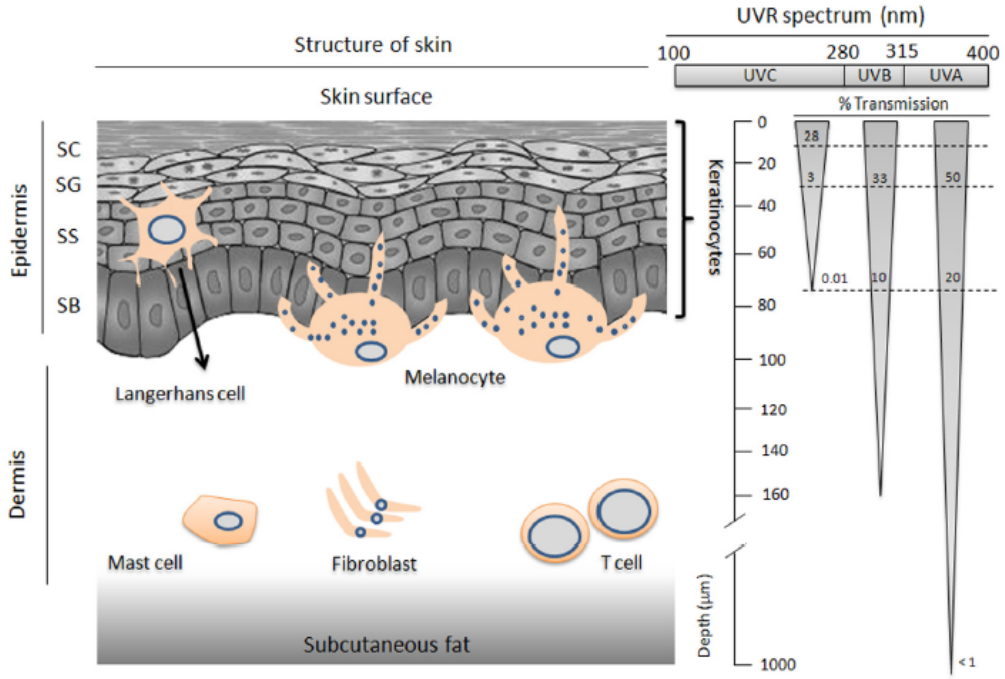


Fig. 3: Penetration of UV light into the skin (Lee et al., 2013)

UVB exposure leads to direct mutations of DNA. The most common mutation appears between neighboring thymidins, resulting in UV-induced cyclobutan pyrimidin dimers (CPDs). Less common is the formation of pyrimidine(6-4)pyrimidone photoproducts (Setlow and Carrier, 1966). These damages occur due to UV-induced deamination of cytosines (C), resulting in uracil, which is commonly not present in DNA. UV-induced DNA damage is largely repaired by nucleotide excision, a DNA repair mechanism of the cell, but sometimes the damage is not detected or the repair fails. In case of C-to-T transition, the DNA repair machinery substitutes uracil with its DNA-equivalent thymine (T) instead of cytosine. Therefore, the repaired DNA still ends up with a mutation (Ikehata and Ono, 2011). Specific proteins of the nucleotide excision repair machinery detect DNA damage and remove defective parts. Parts of this machinery are missing or mutated in the disease xeroderma pigmentosum (XP), leading to a defective nucleotide excision repair. Therefore, XP patients are highly susceptible to UV-induced cancer, starting in childhood, as DNA damage translated largely unbridled into cancer formation

(Knippers, 2001). Besides photochemical damage, UV induces reactive oxygen species (ROS), which cause mutations by oxidation of bases or induction of DNA double strand breaks, a mechanism believed to be more important for UVA than for UVB (Ikehata and Ono, 2011).

3.2.1.2. Common mutations

It was shown that a high number of non-melanoma skin cancers harbor mutations in the gene expressing the transcription factor and tumor suppressor p53 (Brash et al., 1991; Ziegler et al., 1994). Other than *p53* mutations in internal cancers, *p53* mutations in non-melanocytic skin cancer clearly show the so-called UV signature, the before mentioned CPD specific for UV-induced damage (Brash et al., 1991; Greenblatt et al., 1994). One study found 60% of AK containing mutations in *p53* (Ziegler et al., 1994), while another study on invasive SCC reported *p53* mutations in 58% of the cases (Brash et al., 1991). Furthermore, *p53*-mutated keratinocytes are already found in normal-appearing human epidermis exposed to sun light (Jonason et al., 1996).

Wild-type p53 usually gets stabilized by chemical modifications and accumulates upon exposure to UV (Hall et al., 1993) or other cellular stressors like hypoxia or DNA damage (Ozaki and Nakagawara, 2011). P53 in turn can activate the expression of a panel of genes involved in DNA damage repair as well as *p21* expression, leading to cell cycle arrest (Brash, 2006), and genes involved in apoptosis. Whether DNA repair genes or apoptosis genes become activated by p53 depends on the extent of DNA damage (Ozaki and Nakagawara, 2011). Moreover, p53 was found to target *NOTCH1* gene expression, thereby promoting keratinocyte differentiation and tumor suppression (Lefort et al., 2007).

Mutated p53 has a dominant-negative effect on wild-type p53 by forming hetero-oligomers. Furthermore, mutated p53 loses its DNA-binding ability, so that the genes responsible for cell cycle arrest or apoptosis are not expressed anymore upon DNA damage (Ozaki and Nakagawara, 2011). As a result, mutated cells proliferate further in an uncontrolled fashion leading to tumor development and progression.

According to the COSMIC data base, which lists somatic mutations in cancer, there are several other mutations besides *p53* detectable in SCC (Fig. 4) (Cosmic, 2014).

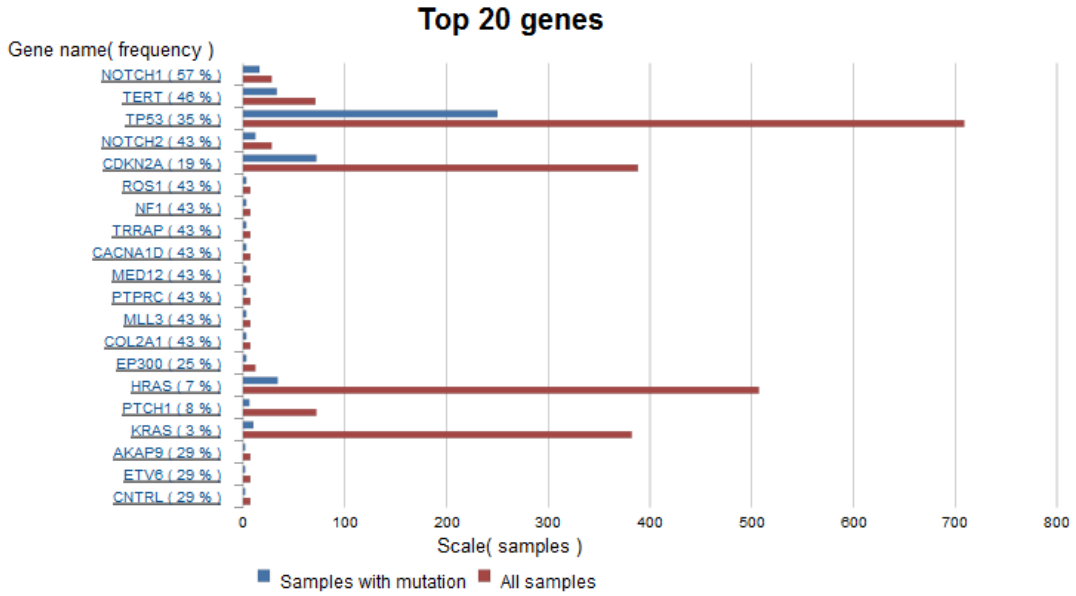


Fig. 4: Frequency of mutations in cutaneous SCC according to the catalogue of somatic mutations in cancer (access date: 2014-12-16)(Cosmic, 2014).

The protein Notch1, capable to induce *p21* expression in keratinocytes, thereby suppressing growth and activating keratinocyte differentiation (Rangarajan et al., 2001) is one of the frequently mutated targets in SCC. Loss of Notch1 prevents keratinocyte differentiation (Moriyama et al., 2008). Tissue-specific Notch1 knock-out in mouse epidermis led to hyperplasia and the development of skin tumors (Nicolas et al., 2003), indicating the important role of Notch1 in keratinocyte differentiation and tumor suppression. Furthermore, Notch1 seems to exert homeostatic control even from the dermal compartment, as mice with impaired mesenchymal Notch1 signaling spontaneously develop SCC (Hu et al., 2012; Proweller et al., 2006).

The *TERT* (telomerase reverse transcriptase) gene encodes for the catalytic part of the enzyme telomerase responsible for the elongation of telomers. TERT usually is only active in self-renewing cells like stem cells. However, it is overexpressed in cancer cells and maintains their infinite proliferation ability. Activating mutations in the *TERT* promoter were found in various cancer types, including SCC, BCC and melanoma (Heidenreich et al., 2014).

The *CDKN2A* (cyclin-dependent kinase inhibitor 2A) gene encodes for the p16 tumor suppressor protein involved in the control of the cell cycle G1 phase. It can bind to the cyclin-dependent kinases 4 and 6, thereby preventing their binding to cyclins in turn and leading to cell cycle arrest (Witkiewicz et al., 2011). Furthermore, p16 expression is associated with induction of cellular senescence (LaPak and Burd, 2014). A mutation in this gene prevents cell cycle arrest in G1.

The *HRAS* (Harvey rat sarcoma viral oncogene homolog) gene belongs to the *ras* oncogene family and encodes for a GTPase. Ras proteins mediate signaling through the ERK1/2 pathway in keratinocytes and participate in proliferation and differentiation processes. Several mouse models show hyperproliferation and tumor development in correlation with increased Ras activity (Drosten et al., 2013).

As mentioned above, normal-appearing skin exposed to sun light, harbors *p53* mutations (Jonason et al., 1996). Accordingly, also AK, the intraepithelial lesion of SCC, shows frequent mutations in this gene. Likewise, AK lesions show a low frequency of *CDKN2A* and *HRAS* mutations (Fig. 5) (Cosmic, 2014).

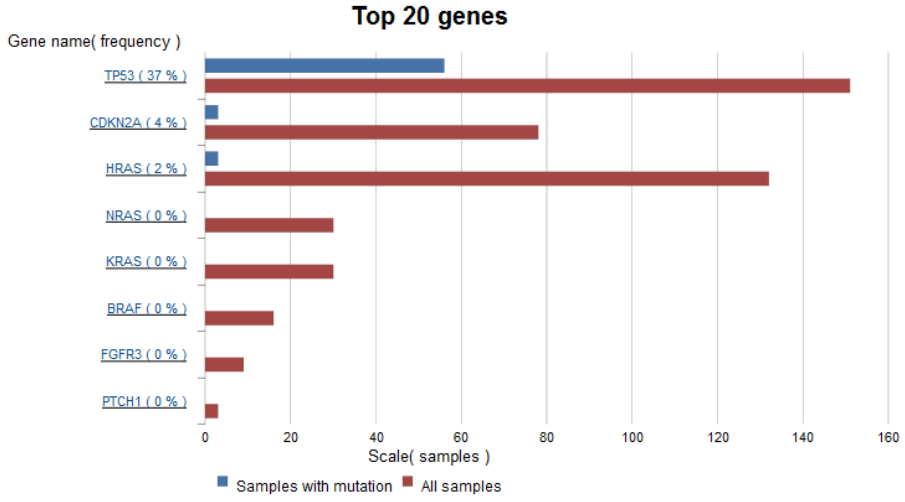


Fig. 5: Frequency of mutations in AK according to the catalogue of somatic mutations in cancer (access date: 2014-12-16) (Cosmic, 2014)

3.2.1.3. Role of immunosuppression

Immunosuppressed patients have a 100-fold higher risk to develop SCC (Stockfleth et al., 2002). As for the general population, the most important risk factor for SCC development is UV damage by sun exposure. In line with this, a study in OTR showed the

prevention of AK and SCC by the regular use of sunscreen with high sun protection factor (SPF) (Ulrich et al., 2009). Furthermore, the intake of immunosuppressive drugs is associated with a higher incidence of SCC. The effect of the immunosuppressive drug cyclosporine A (CsA) on SCC development was studied in OTR, where a lower SCC incidence was seen in patients on low-dose CsA compared to patients on high-dose CsA (Dantal et al., 1998).

Another immunosuppressive drug, azathioprine, generates DNA mutations under the influence of UVA light. The active metabolite of azathioprine, 6-thioguanine, incorporates into the DNA and absorbs UVA light preferentially. Absorption of photons by 6-thioguanine produces reactive oxygen species (ROS) which damage the DNA resulting in an unspecific, diffuse pattern of DNA mutations (O'Donovan et al., 2005). Clinically, a switch from azathioprine to immunosuppressive mTOR inhibitors is recommended to avoid increased mutational load and to benefit from the antiproliferative properties of mTOR inhibition. In line with these experimental observations, a study in OTR revealed a normalized photosensitivity in patients switching from azathioprine to mycophenolate mofetil (Hofbauer et al., 2012).

3.2.1.4. Potential key mediators in SCC development and progression, investigated within the present thesis

Several cell surface receptors, commonly known for their expression on immune cells, were found to be expressed as well on keratinocytes. One of these receptors is the receptor for advanced glycation end products (RAGE). RAGE belongs to the immunoglobulin superfamily of receptors and was first found to bind advanced glycosylation end products (AGEs) (Neeper et al., 1992). Meanwhile, it was also shown to be a receptor for the S100 family of calcium-binding proteins and HMGB1, both playing a role in inflammation. Binding of the ligands to the receptor results in activation of several signaling cascades e.g. MAPK pathways or NFκB pathway (Bierhaus et al., 2005). Besides its role in inflammation, RAGE was found to promote tumor development. Knock-out mice for RAGE become resistant to DMBA/TPA-induced skin cancer (Gebhardt et al., 2008). Furthermore, knockdown of RAGE in human gastric cancer cells leads to inhibition of growth and invasion (Xu et al., 2013) and the Co-expression of RAGE and its ligand HMGB1 is associated with a poor prognostic outcome in patients with prostate cancer (Zhao et al., 2014). In one of our projects we focused on

the impact of the RAGE-S100A8/A9 interaction on human keratinocytes and SCC (see section V.2).

Not only RAGE, but also Toll-like receptor 4 (TLR4) can bind to HMGB1 (Park et al., 2006) and S100A8/A9 (Vogl et al., 2007). Moreover, TLR4 also has other ligands such as lipopolysaccharide A (LPS). TLR4 is expressed by various human tumor cell lines and various human tumors (Oblak and Jerala, 2011), but also on normal cells such as keratinocytes (Song et al., 2002). Therefore, the role of TLR4 in tumor development and progression is controversially discussed (Oblak and Jerala, 2011). A study in colorectal cancer patients showed a correlation between TLR4 expression and poor prognosis (Wang et al., 2010). In melanoma, TLR4 stimulation was associated with induction of cell migration (Goto et al., 2008). Downregulation of TLR4 in breast cancer cells significantly reduced their viability (Yang et al., 2010) while TLR4 knock-out mice are resistant to chemically induced carcinogenesis (Mittal et al., 2010). In contrast, another study showed that TLR4-defcient mice develop more tumors after DMBA treatment (Yusuf et al., 2008). Furthermore, TLR4 expression drops with increasing Gleason score, a grading system to evaluate prostate cancer (Gatti et al., 2009). In our lab, we wanted to delineate the role of TLR4 expression and signaling in keratinocytes and SCC development (see section V.3).

In our project on TLR4 and its involvement in SCC development and progression, we used a blocking antibody for TLR4 and found it to increase keratinocyte proliferation. During our studies it was recognized that TLR4 blocking antibodies cross-react with and bind to Fcγ receptors (FcγR) (Shang et al., 2014). Hence, we were interested whether FcγRs are also involved in the process of SCC development. Commonly, FcγRs are expressed by cells of the immune system and bind specifically to the constant portion of immunoglobulin G (IgG). FcγRs can be divided into three subclasses, FcγRI (CD64), FcγRII (CD32) and FCγRIII (CD16), whereas FcγRI has a high affinity to its ligands while FcγRII and FcγRIII have a low affinity (Ravetch and Kinet, 1991). Moreover, FcγRII has three different isoforms, with FcγRIIA and FcγRIIC being activating receptors and FcγRIIB being an inhibitory receptor, all of them triggering different cellular responses such as proliferation or pro-inflammatory immune responses (Nimmerjahn and Ravetch, 2008). FcγRIIB was previously shown to inhibit proliferation of mast cells

when co-aggregated with the receptor tyrosine kinase c-kit (Malbec et al., 1999). Furthermore, it can cross-link to the B-cell receptor, thereby inhibiting B cell proliferation (Nimmerjahn and Ravetch, 2008). FcγRs were also found expressed on keratinocytes (Cauza et al., 2002; Cowan et al., 1998), but their function on these cells is not known yet. However, several links suggest a connection between FcγRII and keratinocyte proliferation. For example, it has been reported, that senescent keratinocytes show a higher DNA-binding activity for NFκB (Bernard et al., 2004), while repression of NFκB in the epidermis of mice leads to induction of hyperplasia by proliferating keratinocytes (Lewis and Spandau, 2007). Moreover, FcγRII silencing decreases cytosolic IκB degradation, thereby blocking NFκB activation (Liang et al., 2006). As mentioned above, UV light and immunosuppression play a major role in the development of SCC. Accordingly, it was shown that UVB irradiation decreases FcγRII expression on Langerhans-like dendritic cells (Mizuno et al., 2004). All together, the current knowledge on FcγRII and its role in immune cell proliferation, as well as the fact that the function of these receptors has not been investigated yet in the context of SCC, made FcγRII an interesting target in keratinocytes that we further investigated (see section V.0).

The immunosuppressive drug CsA is a calcineurin inhibitor. The fact that CsA-treated patients develop considerably more SCC led to the assumption that impaired calcineurin signaling favors SCC development (Tiu et al., 2006). Indeed, mice with a knock-out for the calcineurin B1 gene *CnB1* showed increased tumor growth compared to wild type mice (Wu et al., 2010). Furthermore, the activating transcription factor 3 (ATF3) was found to be negatively controlled by calcineurin signaling (Wu et al., 2010). Consistently, a study showed that ATF3 is highly expressed in SCC and even higher in metastatic SCC (Kim et al., 2011). In one of our projects we investigated the impact of UVA exposure on ATF3 expression and the potentiation of UVA in combination with CsA (see section V.5).

3.2.2. Prevention of AK and SCC

The most important point in the prevention of AK and SCC is sun-protection. The general population and specifically high-risk groups such as OTR should protect themselves from sun- or more specifically from UV exposure by behavior seeking shade, use of protective clothing and the application of sun screen. Several studies document a

beneficial effect of sunscreen on the reduction of AK and SCC (Naylor et al., 1995; Thompson et al., 1993).

3.2.3. Therapeutic options for AK and SCC – advantages and disadvantages

Current options to treat AK are either topical treatments with 5-fluoruracil, diclofenac with hyaluronic acid, imiquimod, ingenol mebutate, photodynamic therapy (PDT) or radiotherapy for field cancerization or cryotherapy with liquid nitrogen for single lesions (Euroderm, 2011). Invasive SCC usually undergo surgical excision (Motley et al., 2002).

3.2.3.1. Photodynamic Therapy (PDT)

In PDT, a photosensitizer pro-drug is applied topically to the skin followed by exposure to light of a specific wavelength which then in turn activates the photosensitizer and triggers the photodynamic reaction. The generated reactive oxygen species (ROS) are cytotoxic, leading to tumor cell death (Allison and Moghissi, 2013). Furthermore, PDT can lead to systemic, antigen-specific immune responses (Mroz et al., 2010). PDT is given as single treatment or if necessary in repeat cycles. For the procedure of PDT, the topically applied photosensitizer pro-drug needs to incubate on the skin for several hours to convert into protoporphyrin IX before light exposure in tumor cells which makes it cumbersome for the patient (Morton et al., 2013). However, the treatment leads to sustained lesion clearance in 78% to 100% of the patients depending on the photosensitizer used (Szeimies et al., 2010; Tschen et al., 2006).

3.2.3.2. Cryotherapy

Single lesions can be easily treated with cryotherapy. Here, the lesion gets exposed to liquid nitrogen, leading to cell death of both healthy and cancerous cells. In a prospective study, cryotherapy led to a complete response rate of 67.2%. Furthermore, it was shown that prolonged exposure to liquid nitrogen is more effective (Thai et al., 2004). Possible side effects are scar formation or hypopigmentation of the treated area.

3.2.3.3. 5-Fluorouracil (5-FU)

5-fluorouracil (5-FU) is the active compound of a cream for topical treatment of AK. It is a chemotherapeutic agent and interferes with DNA synthesis. It has to be applied daily for several weeks and is therefore laborious for the patient. Furthermore, several side effects can occur while local skin reactions last for several weeks (Euroderm, 2011).

3.2.3.4. Diclofenac

Diclofenac is a cyclooxygenase-2 (COX-2) inhibitor with anti-inflammatory capacity that was also shown to induce apoptosis in neoplastic cells (Fecker et al., 2007). It is used as topical treatment for AK. Diclofenac combined with hyaluronic acid needs to be applied for 60-90 days – twice daily. A meta-analysis of several efficacy studies show clearance rates of 40% compared to 12% for vehicle treatment (Pirard et al., 2005).

3.2.3.5. Imiquimod

Like 5-FU and diclofenac, imiquimod is a topical treatment for AK. It is a Toll-like receptor 7 (TLR7) agonist and therefore stimulates the immune system by activating the NFκB pathway resulting in an Interferon α-dominated cytokine response (Schon and Schon, 2007). Two phase III trials revealed a complete clinical clearance rate of 45.1% after 16 weeks of treatment with two treatments per week (Lebwohl et al., 2004). Imiquimod causes local skin reactions but is overall well tolerated by the patients (Lebwohl et al., 2004). In a randomized study, treatment of AK patients with imiquimod was compared to 5-FU and cryotherapy. Imiquimod reached the highest histological clearance rate with 73% and the lowest recurrence in the 1-year follow-up as well (Krawtchenko et al., 2007).

3.2.3.6. Novel treatment options

Recently, a novel topical therapeutic agent, ingenol mebutate (IM), was approved for the treatment of AK. As it is the core subject of the present thesis, IM will be introduced separately (see section III.4)

3.2.3.7. Surgical excision of the lesion

Since SCC can metastasize, it is important to fully remove them. Small, well-differentiated tumors can be removed by curettage, whereas most SCC will be excised with a safety margin followed by histopathological workup to ascertain the total removal of the cancer (Motley et al., 2002). However, surgical removal of the lesion leads to scar formation.

4. Ingenol mebutate (IM)

4.1. Discovery, isolation and commercialization

Ingenol mebutate (IM) belongs to the family of hydrophobic diterpene esters and is extracted and purified from the sap of the plant *Euphorbia peplus* (Fig. 6).

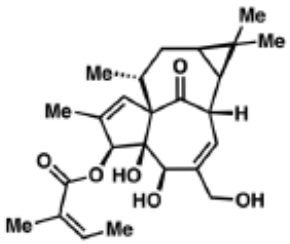


Fig. 6: *Euphorbia peplus* plant (left) (IEWF); chemical structure of ingenol mebutate (right) (Jorgensen et al., 2013).

In a study published in 1988 by Green et al. people stated to use the plant as effective home treatment of cancerous skin lesions and solar keratosis (Green and Beardmore, 1988). Furthermore, the sap of *E. peplus* was shown to have an effect against non-melanoma skin cancer (Ramsay et al., 2011) while IM was shown to be the effective molecule in the sap (Ogbourne et al., 2004). IM was co-developed by the Australian company Peplin Ltd. and LEO Pharma and is now manufactured by LEO Pharma under the trade name Picato®. The drug is registered in the US and Europe as a gel for topical field treatment of AK. Today, IM is extracted from the plant for the production of the drug. However, a new method to chemically synthesize IM was published recently, potentially optimizing the production of the drug and offering the chance to further analyze these compounds for cancer therapy and to progress in the design of new compounds (Jorgensen et al., 2013).

4.2. Therapy

Two different concentrations of IM are available for the treatment of AK depending on the localization of the lesions. For lesions on face and scalp, 0.015% IM gel is used once daily for three consecutive days, while lesions on trunk and extremities, where the epidermis is thicker, are treated with 0.05% IM gel once daily for two consecutive days.

4.3. Pre-clinical Studies

Before the drug was approved for clinical use, several studies on pharmacodynamics, pharmacokinetics and toxicology were performed (European Medicines Agency, 2012). Anti-cancer activity *in vitro* was shown in several cancer cell lines with an LD_{90} of 180-220 µM as well as *in vivo* by curing a panel of mouse and human tumors, induced by subcutaneously injected tumor cells, by three consecutive days of topical treatment (Ogbourne et al., 2004). Furthermore, anti-proliferative effects of IM were shown in colon cancer cells (Benhadji et al., 2008). *In vivo* studies revealed a reduction of skin lesions in chronically UVB-irradiated mouse skin after IM treatment (Cozzi et al., 2012) as well as the effectiveness of IM against a subcutaneously injected mouse SCC cell line (Cozzi et al., 2013). Numerous studies were performed to unravel the mechanism of action of IM in several cancer settings, which will be discussed in detail in a separate section (see section III.4.7).

4.4. Clinical Studies

Several clinical studies were performed with IM gel showing its high efficacy while being well tolerated by the patients. In a randomized, double-blind, vehicle-controlled multi-center study IM gel was tested at different concentrations and turned out to be most effective at 0.05% with complete clinical clearance of 71% of the treated lesions (Siller et al., 2009). In another safety and efficacy study, patients were treated with either placebo gel or 0.015% IM gel for face and scalp or with 0.05% IM gel for trunk and extremities, respectively. For face and scalp the treatment resulted in 42.2% complete clinical clearance of the lesions while 63.9% of the patients had partial clearance of the lesions (i.e. > 75% of lesions) compared to placebo gel with only 3.7% complete and 7.4% partial clearance, respectively. Furthermore, treatment of trunk and extremities resulted in complete clinical clearance of the lesions in 34.1% of all patients (placebo 4.7%) and partial clearance in 49.1% of the patients (placebo 6.9%) (Lebwohl et al., 2012).

Local skin responses like erythema, flaking, scaling, dryness, crusting and itching were observed due to IM gel treatment. These responses were evaluated in a Mean Composite Local-Skin-Response Score and peaked between day 4 and 8 of the study (Lebwohl et al., 2012). Furthermore, there was low incidence of adverse events, and no serious adverse events occurred due to the treatment (Lebwohl et al., 2012; Siller et al., 2009) .

4.5. Advantages over other treatments

Other than PDT or cryotherapy, topical treatments like IM can be applied by the patients themselves at home, providing convenience. Compared to other topicals like 5-FU, diclofenac or imiquimod with their prolonged application time, IM gel only needs to be applied for two or three days (Fig. 7), facilitating adherence to treatment for the patients.

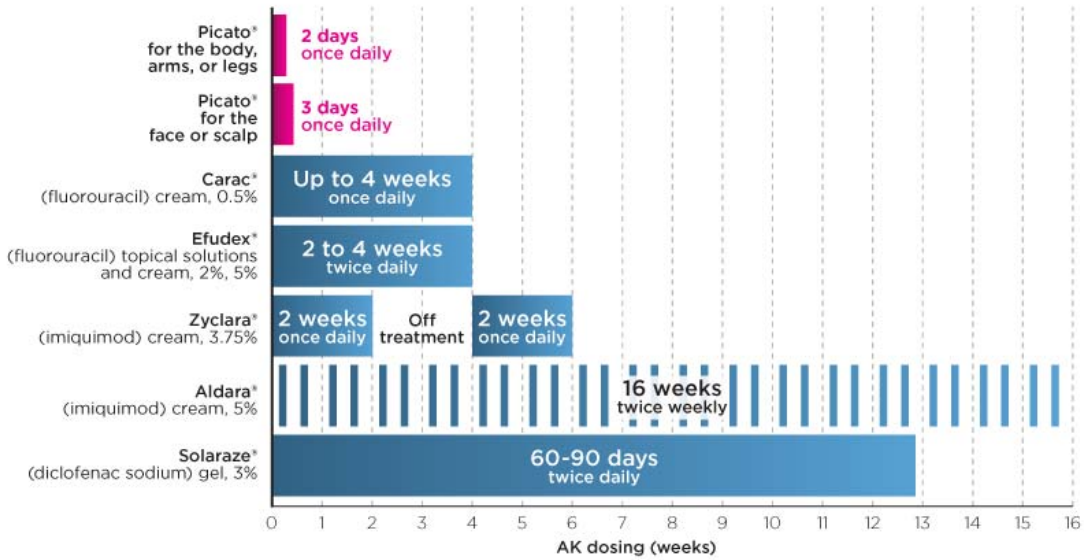


Fig. 7: Treatment schedule for topical AK treatments (LEOPharma, 2013).

Furthermore, the local skin reaction starts rapidly a few hours after IM treatment, peaking at day 4. Local skin responses generally resolved within two to four weeks (Lebwohl et al., 2012), resulting in limited down time for the patients. The absence of systemic effects is an advantage over other topicals such as imiquimod.

4.6. Off-label use

IM gel is registered for treatment of AKs. However, off-label use in other skin diseases has been reported. A randomized phase IIa trial investigated safety and efficacy in superficial BCC. The treatment with 0.05% IM gel on two consecutive days resulted in complete clinical and histological clearance in 63% of the patients (Siller et al., 2010).

A case report on molluscum contagiosum, a viral skin infection in children, showed effectiveness of 0.015% IM gel. The patient was treated for 6 days, resulting in clearance of the lesions (Javed and Tyring, 2014).

Furthermore, another case study reports the complete clinical clearance of a recurrent *in situ* melanoma after treatment with 0.015% IM gel on three consecutive days.

Histological analysis revealed no residues of melanoma while the 6-month follow-up showed no recurrence (Mansuy et al., 2014).

In four cases, actinic cheilitis was treatment with 0.015% IM. The treatment led to clinical improvement in two patients but was not successful in the other two (Barrado Solis et al., 2014).

Recently, 0.05% IM was used to treat *in situ* SCC and seborrheic keratosis. While the effect on seborrheic keratosis was limited (15% clinical clearance), IM showed a clinical clearance rate for *in situ* SCC in 64% of the cases, while the histological clearance rate was 36% (Rosen, 2014).

4.7. Mechanism of Action

Various studies were performed to discover the mechanism by which IM elicits its effect. In a panel of mouse and human cell lines the anti-cancer activity was shown by acute cytotoxicity assays. Small tumors resulting from subcutaneous tumor cell injections into mice could be cured as well. Furthermore, it was demonstrated, that the treatment induces necrosis (Cozzi et al., 2013; Ogbourne et al., 2004). Another study revealed that IM interacts with protein kinase C (PKC) (Kedei et al., 2004), followed by the finding that IM efficacy correlates with PKCδ expression in myeloid leukemia cells (Hampson et al., 2005). In IM-sensitive melanoma cells, IM induced cell cycle arrest and over-activated the Mitogen-Activated Protein Kinase (MAPK) signaling pathway (Cozzi et al., 2006). IM treatment of a colon cancer cell line induced various signaling pathways, including PKC, Raf1, ERK1/2, JNK, p38 and PTEN (Serova et al., 2008).

With regard to the immune system, IM treatment leads to the recruitment of neutrophils possibly by inducing inflammatory cytokines or PKC-mediated activation of endothelial cells (Challacombe et al., 2006; Hampson et al., 2008). Besides neutrophil infiltration, IM treatment caused hemorrhage and mast cell activation in mice (Li et al., 2010). Overall, there is a dual mechanism of action of IM considered (Fig. 8), where a rapid necrosis of the lesion within hours, is followed by the activation of the innate immune system (Rosen et al., 2012). While one study in mice reported an increase of anti-tumor antibodies due to activation of B cells leading to neutrophil-mediated antibody-dependent cellular cytotoxicity (ADCC) (Challacombe et al., 2006), there is no evidence that this mechanism is also relevant in humans.

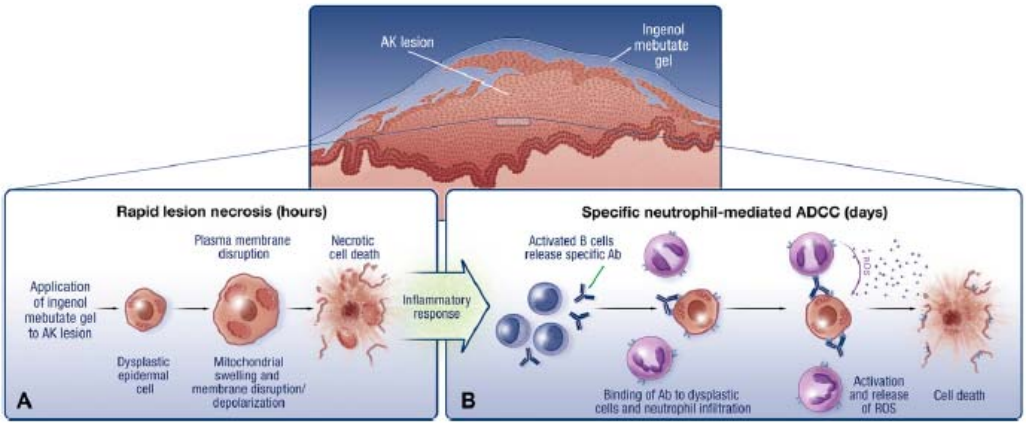


Fig. 8: Dual mechanism of action of IM (Rosen et al., 2012). A) Rapid necrosis of keratinocytes. B) neutrophil-mediated ADCC.

So far, only one study reports an effect on keratinocytes, the main target of IM. The authors studied the effect of clinical IM concentrations on cell cultures showing acute cytotoxicity and disruption of the mitochondrial network (Stahlhut et al., 2012).

5. Protein kinase C (PKC)

Protein kinase C (PKC) belongs to the family of serine/threonine kinases and can be divided into three subclasses: classical, novel and atypical PKC isoforms. The classical isoforms α, βI, βII and γ (Coussens et al., 1986; Parker et al., 1986) were shown to be calcium sensitive and need additional activators like diacylglycerol or phorbol esters for their activation (Arcoleo and Weinstein, 1985). The novel isoforms include δ, ε, η and Θ (Ono et al., 1987) and are calcium-independent. The atypical isoforms are PKC ζ and ι/λ (Bacher et al., 1992; Osada et al., 1992), which only require phosphatidylserine for activation (Denning, 2004). Not all of these isoforms are expressed in keratinocytes. Among the classical isoforms, only PKCα is present, while most novel isoforms (PKCδ, PKCε, PKCη) and the atypical isoform PKCζ are also expressed (Denning, 2004).

The role for PKCα in keratinocytes is controversial. Several reports show an involvement of PKCα in keratinocyte differentiation. For example, Yang et al. demonstrated an important role of PKCα in promoting keratinocyte differentiation by showing that PKCα knock-down decreases involucrin promoter activity (Yang et al., 2003). Furthermore, Jerome-Morais et al. were able to inhibit keratinocyte proliferation by overexpressing a constitutively active variant of PKCα (Jerome-Morais et al., 2009).

The role of PKCδ has been investigated in various cell types and tissues before. In this context, PKCδ was found to be involved in mediating cell differentiation and inhibition of proliferation (Bowles et al., 2007; Hernandez-Maqueda et al., 2013; Papp et al., 2004; Zhang et al., 2014). For keratinocytes, this was specifically shown in a study of Hernandez-Maqueda et al. (Hernandez-Maqueda et al., 2013), thereby supporting its role as a tumor suppressor. In line with this, Costa et al. reported a loss of PKCδ in human SCC biopsies. However, 75% of all stained cases were still positive for PKCδ compared to 100% positivity in normal human skin samples (D'Costa et al., 2006). Moreover, TPA-mediated keratinocyte differentiation requires PKCδ, confirming its role in the differentiation process (Adhikary et al., 2010).

Other reports, however, show opposing roles for PKCα and PKCδ. One study indicates that PKCδ enhances the promoter activation of the involucrin gene, a marker for keratinocyte differentiation, while PKCα has inhibiting effects (Deucher et al., 2002).

PKCε overexpressing mice are more sensitive to SCC development induced by both TPA (Wheeler et al., 2003) and UV (Wheeler et al., 2005). Furthermore, TPA- or UV-treated PKCε transgenic mice have a lower survival rate than WT control mice (Verma et al., 2006). However, PKCε was found to be lower in expression both in tumors of PKCε transgenic mice and in SCC patient biopsies. The reason may be that PKCε mediates its tumorigenic effect by inducing the secretion of growth factors stimulating the actual tumor cells (Wheeler et al., 2005).

PKCη also plays a role in the process of differentiation as Ueda et al. reported the PKCη-dependent upregulation of transglutaminase 1 gene expression, another marker for terminal differentiation in keratinocytes (Ueda et al., 1996). Along with that, PKCη also positively influences involucrin gene expression (Takahashi et al., 1998).

PKCζ was shown to be more activated in head and neck SCC (HNSCC) compared to normal tissue, whereas its inhibition led to a decreased cell proliferation of HNSCC cell lines (Cohen et al., 2006).

6. MAPK signaling

6.1. MAPK pathways

The mitogen-activated protein kinase (MAPK) family is involved in a great number of cellular processes. Transmembrane receptor tyrosine kinases (RTKs) transmit extracellular stimuli like growth factors, cytokines or stress factors by recruiting Ras- or Rho family members, upstream of the MAPK cascades. These in turn activate the first module of the downstream MAPK cascade by phosphorylation, in turn activating the next downstream module. Four canonical MAPK pathways are known: ERK1/2 pathway, JNK1/2/3 pathway, p38 pathway and ERK5 pathway (Fig. 9). Each of the pathways induces various cellular responses, depending on their downstream targets (Pritchard and Hayward, 2013). ERK1/2 are commonly known to be activated by growth factors resulting in the regulation of several cellular processes like proliferation, differentiation or cell survival (Deschenes-Simard et al., 2014). The JNK pathway is involved in regulation of survival and apoptosis and gets activated mainly by stress factors (Pritchard and Hayward, 2013). The p38 cascade is activated upon environmental stress stimuli and inflammatory cytokines and plays a role in regulating stress responses, but was also shown to be involved in apoptosis. To date, 4 isoforms of p38 (α, β, γ, δ) have been identified (Koul et al., 2013). ERK5 signaling is triggered by stress stimuli and growth factors, therefore also being involved in proliferation, migration and cell survival (Nithianandarajah-Jones et al., 2012). Furthermore, there is a cross-talk between the 4 branches of MAPK signaling (Pritchard and Hayward, 2013).

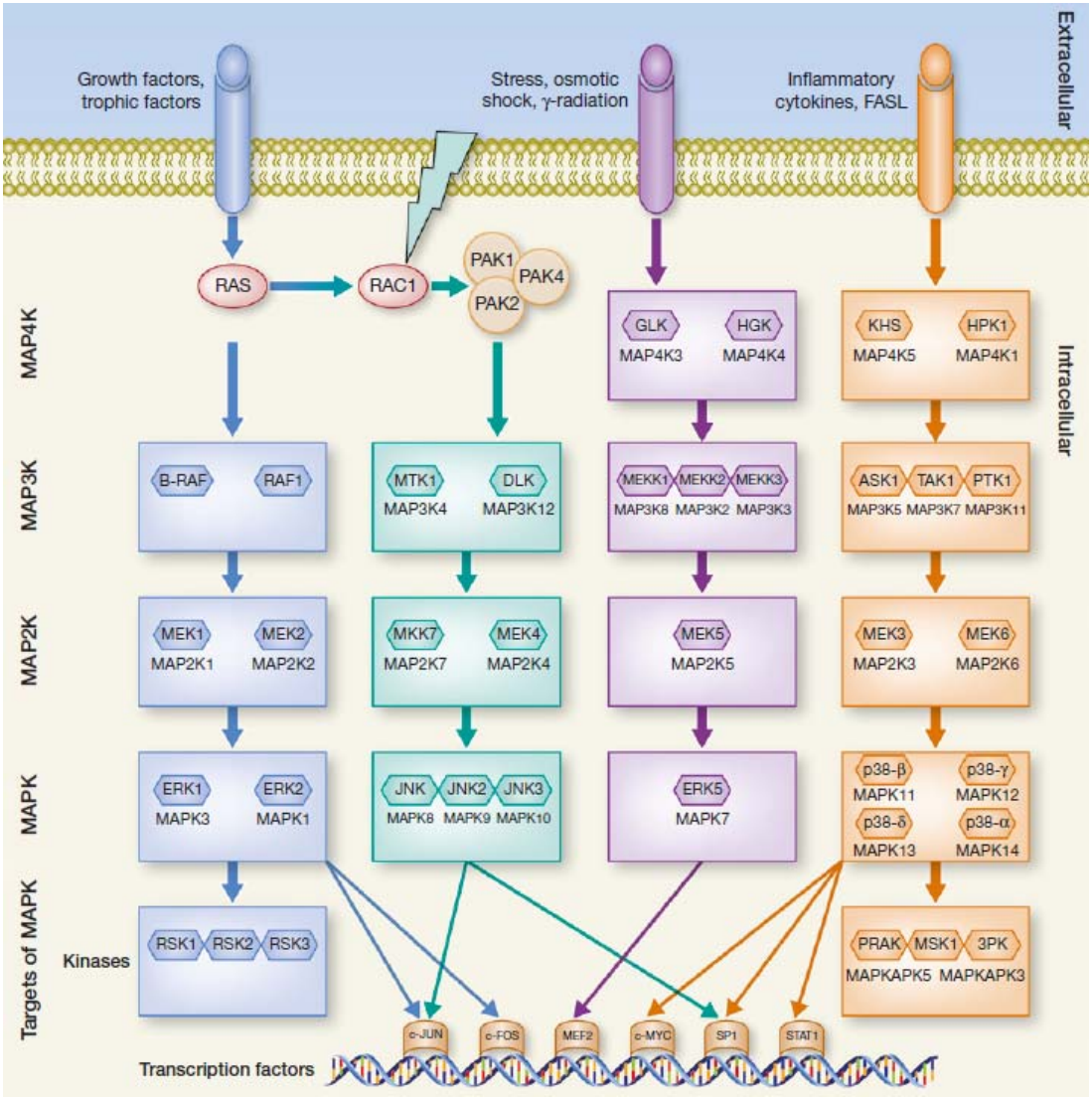


Fig. 9: Overview of canonical MAPK signaling pathways (Pritchard and Hayward, 2013).

6.2. MAPK signaling in cancer

Mutations in the MAPK signaling cascades lead to disruption of important cellular regulation processes, resulting in cancer development. Common alterations in the ERK1/2 signaling pathway are mutations of Ras or Raf that lead to constitutively active signaling, thereby promoting proliferation (Pritchard and Hayward, 2013), whereas MEK or ERK mutations were not shown to play a role in cancer (Deschenes-Simard et al., 2014). As mentioned above, *RAS* is mutated in some SCCs (Cosmic, 2014). The ERK1/2 pathway is generally considered to promote tumor cell growth (Gioeli et al., 1999). Especially for melanoma cells, it is well known that mutations in the *NRAS* and *BRAF*

gene cause permanent activation of the pathway (Davies et al., 2002; Padua et al., 1985). Moreover, increased ERK phosphorylation was found to correlate with poor outcome in colon cancer (Schmitz et al., 2007). Conversely, there are several reports showing increased ERK phosphorylation and activation as beneficial markers, e.g. in clear-cell renal cell carcinoma (Lee et al., 2009) or endometrial cancer (Mizumoto et al., 2007). Furthermore, ERK activation can suppress tumor growth by promoting selective protein degradation (Deschenes-Simard et al., 2013) and serves as a marker for improved outcome in prostate and breast cancer patients (Malik et al., 2002; Svensson et al., 2005).

The role of JNK is controversially discussed as well. On the one hand, several mutations of the JNK pathway are found in human cancers. On the other hand, JNK was shown to be essential for stress-induced apoptosis. Additionally, the role of JNK depends on the duration of activation and on the isoform (Tournier, 2013).

P38 has tumor-suppressive effects by activating p53 and can regulate cell cycle arrest (Koul et al., 2013). Furthermore, p38 responds to chemotherapy and stimulates apoptosis (Bradham and McClay, 2006). Consistently, p38α activity is downregulated in liver cancer. However, in other cancers p38α activity was found to be upregulated (Koul et al., 2013).

7. Interleukin decoy receptors

7.1. IL1R2

Interleukin 1 receptor 2 (IL1R2) belongs to the IL1 receptor family which currently contains 11 different IL1 receptors. Some of them bind IL1 but others also bind to IL18, IL33, IL36 or IL37 (Boraschi and Tagliabue, 2013). Unlike the other receptors, IL1R2 has a very short cytoplasmic domain, and furthermore exists in a soluble form which is produced by proteolytic cleavage by matrix metalloproteases (MMPs). It has a high binding affinity to IL1β as well and binds to IL1α and IL1Ra with low affinity. All these findings point to a decoy function of the receptor, preventing the binding of IL1 to the signal transducing IL1 receptor 1 (Boraschi and Tagliabue, 2013; Colotta et al., 1994). The binding of IL1 to IL1R2 can be reverted by caspase-1 which can cleave the receptor resulting in IL1 release (Garlanda et al., 2013).

IL1R2 was found to be expressed on basal keratinocytes and is upregulated in psoriasis. Furthermore, IL1R2 is upregulated in several tumors like prostate cancer or adenocarcinoma. In contrast, it is little expressed in endometriosis and endometroid ovarian cancer (Garlanda et al., 2013). Consistently, a study in mice showed that soluble IL1R2 can inhibit endometrial tissue growth and decrease the expression of the anti-apoptotic gene and Bcl2 (Khoufache et al., 2012).

7.2. IL13RA2

Interleukin 13 receptor α2 (IL13RA2) was first described in mice and found to bind IL13 with high affinity, but does not mediate a cellular response (Donaldson et al., 1998). Therefore, IL13RA2 was commonly considered to be a decoy receptor. Besides the membrane bound form that can also be present intracellularly (Daines et al., 2006), a soluble variant of the protein was detected (Zhang et al., 1997). It was shown that MMP8 cleaves IL13RA2 and thereby contributes to its solubilization (Chen et al., 2008). One report reveals, that IL13RA2 and IL13 get internalized after binding and that the intracellular domain of IL13RA2 is responsible for this internalization (Kawakami et al., 2001b). IL13RA2 is overexpressed in some cancers, e.g. glioma (Bernard et al., 2001). To delineate the role of IL13RA2 in cancer, IL13RA2 was overexpressed in breast cancer cells and pancreatic cancer cells. *In vitro* these cells did not differ in proliferation behavior. However, when injected into athymic nude mice, IL13RA2-overexpressing breast cancer cells showed reduced tumor growth compared to vehicle transfected cells,

while mice injected with IL13RA2-overexpressing pancreatic tumor cells did not show tumor development at all (Kawakami et al., 2001a). Investigation of the promoter of the IL13RA2 gene suggested several putative binding sites for certain transcription factors like c-Jun, c-Fos, AP2, GABP or OCT1 (Wu and Low, 2003).

8. Aim of the thesis

The recent work of our group is focused on the molecular aspects of development, progression and therapy of cutaneous squamous cell carcinoma (SCC) and its intraepithelial form actinic keratosis (AK).

The main project of this PhD thesis addresses the investigation of the mechanism of action of ingenol mebutate (IM) in healthy and malignant keratinocytes. Although IM is registered for treatment of AK, its exact mechanism of action is not fully understood. Several studies show the effectiveness of IM on various cancer cell lines and, besides the cytotoxic effect, an involvement of the immune system. However, there is only one report studying the effect on SCC cell lines and keratinocytes, using high IM concentrations leading to acute cytotoxicity and mitochondrial network disruption (Stahlhut et al., 2012). In the present project, the direct effect of IM as well as involved signaling pathways were investigated on primary healthy and cancerous keratinocytes as well as on human skin explants (see section V.1).

Other projects of our group address the development of SCC, more specifically the investigation of factors that play a role in mediating the transformation from healthy keratinocytes to malignant cells. We found several cell surface receptors being involved in regulating the process of keratinocytes proliferation and differentiation. In three separate manuscripts we describe the role of RAGE, TLR4 and CD32 in keratinocytes and SCC (see sections V.2, V.3, V.0).

Furthermore, our group is interested in the SCC development in the high-risk patient group OTR. Therefore, we investigated the impact of the immunosuppressive drug CsA in combination with UVA on keratinocytes and skin explants (see section V.5).

IV. Materials and Methods

1. Materials

1.1. Cell lines and primary cultures

SCC12 cell line:

originally isolated from SCC of facial skin of a 60-year old male kidney-transplant recipient (Rheinwald and Beckett, 1981)

SCC13 cell line:

originally isolated from SCC of facial skin of a 56-year old female (Rheinwald and Beckett, 1981)

HaCaT cell line:

spontaneously immortalized keratinocyte cell line, originally isolated from the back skin of a 62-year old male (Boukamp et al., 1988)

Primary keratinocytes:

isolated from healthy skin obtained from mammary and abdominal surgery at the University Hospital Zurich

Primary SCC cells:

isolated from SCC specimen, excised at the Department of Dermatology at the University Hospital Zurich

Melanoma cells:

isolated from melanoma biopsies taken at the Department of Dermatology at the University Hospital Zurich

Fibroblasts:

isolated from healthy skin obtained from mammary and abdominal surgery at the University Hospital Zurich

Human skin explants:

obtained from mammary and abdominal surgery at the University Hospital Zurich

Human SCC punch biopsies:

obtained from totally excised SCC at the Department of Dermatology at the University Hospital Zurich

Institutional board approval for the use of human tissue was granted; all donors signed written informed consent forms in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (ethical approval number EK647). All samples were obtained from the University Hospital Zurich.

1.2. Cell culture

Antibiotic-Antimycotic (100x)	Gibco® (15240-096)
Bovine Pituitary Extract (BPE)	Gibco® (37000-015)
CnT07 and basal medium	CELLnTEC (CnT-07)
Dulbecco's Modified Eagle Medium (DMEM) + 4.5g/l D-Glucose + L-Glutamine + Pyruvate	Gibco® (41966052)
Epidermal Growth Factor 1-53 (EGF 1-53)	Gibco® (37000-015)
Fetal Bovine Serum Gold	PAA (VWR: 95025-540)
Keratinocyte serum-free medium (K-SFM)	Gibco® (10744-019)
OPTI-MEM®	Gibco® (31985-070)
PBS Dulbecco without calcium	Merck Millipore (BSS-1005-B)
Plasmocin™	InvivoGen (ant-mpp)
RPMI 1640	Gibco® (11875-093)
Trypsin/EDTA Solution	Merck Millipore (SM-2003-C)

1.3. Transfection agents

Gene Carrier-1™	Epoche (31-00110)
INTERFERin	Polyplus, Chemie Brunschwig AG (409-50)
Lipofectamine2000	Invitrogen (11668-027)

1.4. RNA and DNA constructs

1.4.1. siRNA

siCtrl	Qiagen (1027310)
target sequence 5'-AATTCTCCGAACGTGTCACGT-3'	
siPKCα #3	Qiagen (SI00042238)
target sequence 5'-CAAGACGTCTTTGGAATCCAA-3'	
siPKCα #5:	Qiagen (SI00301308)

target sequence 5’-AACCATCCGCTCCACACTAAA -3’

siPKCδ #1 Qiagen (SI00086443)

target sequence 5’-CCGGGACACTATATTCCAGAA-3’

siPKCδ #7 Qiagen (SI00301329)

target sequence 5’-AACTCTACCGTGCCACGTTTT-3’

siPKCδ #11 Qiagen (SI02660539)

target sequence 5’-CAGCAGCAAGTGCAACATCAA-3’

siIL1R2 #2 Qiagen (SI00066304)

target sequence 5’-CACGCCAGGAATATTCAGAAA-3’

siIL1R2 #4 Qiagen (SI00066318)

target sequence 5’-AAGACTGACAATCCCGTGTAA -3’

siIL13RA2 #1 Qiagen (SI00013426)

target sequence 5’-CAGGATATGGATTGCGTATAT-3’

siIL13RA2 #5 Qiagen (SI03036397)

target sequence 5’-AAGGTGAAGACCTATCGAAGA-3’

1.4.2. Primer

36B4 fwd: 5’-GCAATGTTGCCAGTGTCTGT-3’

36B4 rev: 5’-GCCTTGACCTTTTCAGCAAG-3’

C15orf48 5’-CCCACCAGGCGATCAATACT-3’

C15orf48 rev: 3’-AGGGAATGAGTTCCTTCCTTTTCA-3’

CFOS fwd: 5’-GGGGCAAGGTGGAACAGTTA-3’

CFOS rev: 5’-AGTTGGTCTGTCTCCGCTTG-3’

DEFB4A fwd: 5’-TGGTGAAGCTCCCAGCCATC-3’

DEFB4A rev: 5’-ATACCACCAAAAACACCTGGAAGA-3’

EGR1 fwd: 5’-ACCTGACCGCAGAGTCTTTT-3’

EGR1 rev: 5’-GAGTGGTTTGGCTGGGGTAA-3’

IL13RA2 fwd: 5’-GCGGGGAGAGAGGCAATATC-3’

IL13RA2 rev: 5’-AGCATCCGATAGCCAAGCAA-3’

IL1R2 fwd: 5’-CAGCTTCTCTGGGGTCAAGA-3’

IL1R2 rev: 5’-CGTGGCCCCTCGGTCA-3’

MMP1 fwd: 5’-CTGGCCACAACTGCCAAATG-3’

MMP1 rev: 5’-CTGTCCCTGAACAGCCCAGTACTTA-3’

PKCδ fwd: 5’-CTGCAAGAAGAACAATGGCAAG-3’

PKCδ rev: 5'-ATCCACGTCCTCCAGGAAATACT-3'
S100A7A fwd: 5'-AATACACCGGACGTGATGGC-3'
S100A7A rev: 5'-ATGCCCTTTTTGTCACAGGC-3'
SPRY2 fwd: 5'-TGCACATCGCAGAAAGAAGAG-3'
SPRY2 rev: 5'-AGAACACATCTGAACTCCGTGA-3'

All primers were designed with Primer Blast (NCBI) and ordered at Microsynth, Switzerland.

1.5. Antibodies

1.5.1. Primary antibodies

anti-ERK (p42/44)	Cell Signaling (#9102)
anti-JNK	Cell Signaling (#9252)
anti-p38	Cell Signaling (#9212)
anti-IL13RA2	Abcam (ab55275)
anti-IL1R2	R&D (MAB2631) or (sc-376247)
anti-phospho-ERK (pp42/44)	Cell Signaling (#9101)
anti-phospho-JNK	Cell Signaling (#9251)
anti-phospho-p38	Cell Signaling (#9211)
anti-phospho-PKCα/βII (T638/641)	Cell Signaling (#9375)
anti-phospho-PKCδ (T505)	Cell Signaling (#9374)
anti-phospho-PKCδ (Y311)	Cell Signaling (#2055)
anti-PKCδ	Cell Signaling (#9616)
anti-RAGE	Millipore (MAB5328)
anti-β-Actin	Santa Cruz Biotechnology (sc-47778)

1.5.2. Secondary antibodies

Goat anti-rabbit IgG (H&L) HRP	Cell Signaling (#7074)
Rabbit anti-goat IgG HRP	Santa Cruz Biotechnology (sc-2768)
Rabbit anti-mouse IgG (H&L) HRP	Abcam (ab6728)

1.6. Arrays and kits

BrdU assa kit	Millipore (2752)
Dako REAL Detection System	Dako (K5005)
Dc Protein Assay	BioRad (500-0116)
EndoFree Plasmid Maxi Kit	Qiagen (12362)
GoScript™ Reverse Transcription System	Promega (A5000)
PathScan® Intracellular Signaling Array Kit	Cell Signaling (#7323)
RNeasy® MiniElute cleanup Kit	Qiagen (74204)
SurePrint G3 Human Gene Expression array (8x60K v2)	Agilent (G4851B)

1.7. Drugs

12-O-Tetradecanoylphorbol-13-acetate (TPA)	Sigma (P1585)
AEB071 (PKC inhibitor)	Selleckchem (S2791)
GSK1120212 (MEK inhibitor)	Cellagen Technology (C4112-5)
Ingenol Mebutate gel	LEO Pharma
Ingenol Mebutate in DMSO	LEO Pharma
SB203580 (p38 inhibitor)	Cell Signaling (#5633)
SCH772984 (ERK inhibitor)	ChemScene (CS-1421)
SP600125 (JNK inhibitor)	Santa Cruz (sc-200635)

1.8. Chemicals

30% Acrylamide/Bis Solution 19:1	BioRad (161-0154)
β-Mercaptoethanol	Sigma (M6250)
Agarose	Sigma (A9539)
Ammoniumpersulfate (APS)	Sigma (A3678)
Bovine serum albumin (BSA)	Sigma (A9418)
Bromphenol blue	Fluka (18030)
Chloroform	Sigma (472476)
Complete Protease Inhibitor Cocktail	Roche (04693116001)
Dimethyl Sulfoxide (DMSO)	Sigma (D4540)
Dispase II	Roche (04942078001)
ECL solution	Amersham (RPN2106)
Ethanol	Sigma (02860)

FastStart Universal SYBR Green Master (ROX)	Roche (04913914001)
Formaldehyd (4%, buffered)	Kantonsapotheke Zurich
Formic acid	Sigma (F0507)
Glycine	Sigma (G7126)
Glycerol	Fluka (49783)
Hydrochloric acid (HCl)	Fluka (84415)
Isopropanol	Fluka (34965)
Methanol	Sigma (32213)
MTT (Methylthiazolyldiphenyl-tetrazolium bromide)	Sigma (M2128)
PageRuler Prestained Protein Ladder	Thermo Scientific (26617)
PhosSTOP Phosphatase Inhibitor Cocktail	Roche (04906845001)
ProLong® Gold Antifade Mountant with DAPI	Molecular Probes (P-36931)
Protein Ladder	Geneaid (PL0245)
Skimmed milk powder	Coop, Zurich
Sodium chloride	Sigma (S9625)
Sodium dodecyl sulfat (SDS)	Sigma (71725)
Tetramethylethylenediamine (TEMED)	Sigma (T9281)
TritonX100	Thermo Scientific (85112)
Trizma® base	Sigma (T1503)
TRIzol® Reagent	Ambion® (15596018)
Tween 20	Sigma (93773)

1.9. Buffers

1.0M Tris pH 6.8

121.14 g Trizma® base
Adjust pH to 6.8 with HCl
Add H_2O to 1 l

1.5M Tris pH 8.8

181.71 g Trizma® base
Adjust pH to 8.8 with HCl
Add H_2O to 1 l

3x Laemmli buffer (for 10 ml):

2.4 ml 1M Tris (pH 6.8)

3 ml 20% SDS

3 ml Glycerol

1.6 ml β-Mercaptoethanol

6 mg Bromphenol blue

10x SDS-PAGE running buffer

144 g Glycine

30 g Trizma® base

50 ml 20%SDS

Add H_2O to 1 l

→ use: 100 ml 10x Running buffer + 900 ml H_2O

10x TBS

60.6 g Trizma® base

87.6 g Sodium chloride

Adjust pH to 7.6 with HCl

Add H_2O to 1 l

10x Transfer buffer

144 g Glycine

30 g Trizma® base

Add H_2O to 1 l

→ use: 100 ml 10x Transfer buffer + 800 ml H_2O + 100 ml Methanol

Blocking solution for immunofluorescence

PBS

0.1% Tween20

1% BSA

Blocking solution for WB

TBS/T

5% skimmed milk powder

PBS/T (washing buffer immunofluorescence)

1000 ml PBS

1 ml Tween 20

Permeabilization solution for immunofluorescence

PBS

0.1% TritonX1000

RIPA buffer — Cell Signaling (#9806)

+ 1mM PMSF before use — Sigma (P7626)

Stripping buffer

15 g glycine

1 g SDS

10 ml Tween 20

Adjust pH to 2.2 with HCl

Add H_2O to 1 l

Solution A (for MTT assay)

95% Isopropanol

5% Formic acid

Solution B (for MTT assay)

10% SDS in PBS

TBS/T (washing buffer for WB)

100 ml 10x TBS

900 ml H_2O

0.1% Tween 20 (1 ml)

1.10. Consumables

Cell culture flasks — Falcon

Cell scraper — Nunc, VWR International AG

	(734-2132)
Combitips (10 ml, 5 ml, 1 ml)	Eppendorf (089.677, 069.455, 069.439)
Cover slips (18x18 mm)	Menzel GmbH (BB018018A1)
Cryo tubes	Nalgene, VWR International AG (NALG5000-0020)
Eppendorf tube (1.5 ml)	Eppendorf (030 121.589)
Falcon tubes (15 ml, 50 ml)	Corning Life Science (352096, 352070)
Glass slides Superfrost Plus	Thermo Scientific (J1810AMNZ)
Hyperfilm ECL	Amersham Bioscience (28-9068-37)
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems
PVDF membrane	BioRad(162-0177)
Whatman paper	Whatman (514-8013/3030917)

1.11. Devices

Film developer	
Microscope CLSM Leica SP5	Leica
Mini Protean® 3 Cell (electrophoresis system)	BioRad
Mini Trans-Blot® Cell (transfer system)	BioRad
Plate reader	Tecan
Power supply	BioRad
ViiA7 qPCR machine	Applied Biosystems

1.12. Software

Adobe Acrobat XI Pro	Adobe
Adobe Illustrator CS6	Adobe
Endnote X6	Thomson Reuters
GeneGo	Thomson Reuters
GraphPad Prism 5	GraphPad Software
Image J 1.47t	National Institute of Health
Microsoft office excel 2010	Microsoft

2. Methods

2.1. Isolation of primary cells

Healthy human skin was obtained from mammary and abdominal surgery at the University Hospital Zurich (USZ). Melanoma cells were isolated from patient biopsies at the USZ. All donors signed written informed consent forms according to the ethical approval EK647. Human primary SCC cells were isolated from punch biopsies taken from SCC surgical excisions at the Department of Dermatology of the University Hospital Zurich. Institutional board approval for the use of human tissue was granted.

Healthy human skin pieces (approx. 0.5-1 cm^2) or SCC punch biopsies (Ø 3-5 mm) were incubated over night at 4°C in CnT07 medium containing antibiotics and 10 mg/ml dispase. Afterwards, the epidermis was separated manually and incubated in pre-warmed trypsin for approx. 5 min. Then, a cell scraper was used to scratch the epidermis for releasing the keratinocytes. The epidermis was then washed with CnT07 medium under repeated scratching. The keratinocytes were then centrifuged at 1500 rpm for 5 min. The pellet was re-suspended in CnT07 medium and transferred to a culture flask. For punch biopsies as starting material, the cells were transferred to a 6-well. Medium was changed every two days until the cells reached 80-90% confluence and could be split for the first time.

Melanoma cells and fibroblasts were kindly provided by the research group of Prof. Dummer/Prof. Levesque.

2.2. Culture of cells and skin explants

2.2.1. Cell lines

SCC12 cells were cultured in Keratinocyte serum-free medium (K-SFM) supplemented with BPE and EGF. HaCaT cells and SCC13 cells were cultured in DMEM/10%FCS. Plasmocin was added to avoid mycoplasma contamination.

All cell lines were split between 1:3 and 1:10 when reaching about 80-90% confluence.

2.2.2. Primary cell cultures

Primary keratinocytes and primary SCC cells were cultured in CnT07 medium. Melanoma cells and fibroblasts were cultured in RPMI/10% FCS. All cell types were split 1:2 when reaching 80-90% confluence.

2.2.3. Skin explants

Explants from healthy human skin and human cutaneous SCC were cultured on semi-solid culture medium containing CnT07 medium with antibiotics. The semi-solid culture medium was prepared by first dissolving and heating 0.5g agarose powder in H_2O. 1 ml of the agarose solution was then added to 9 ml of pre-warmed CnT07 medium containing antibiotics and transferred to ∅ 3.5 cm culture dishes. After the medium turned solid, the skin explants were put on top.

2.3. Transfection

2.3.1. siRNA

Cells were seeded on 24-well plates or 6-well plates at 30% confluence. The transfection mix was prepared in basal keratinocyte medium as indicated in table 1.

Tab. 1: Preparation of siRNA transfection mix per reaction with INTERFERin.

	6-well plate	24-well plate
Basal medium	400 µl	100 µl
INTERFERin	2 µl	0.5 µl
siRNA (20µM)	2 µl	0.5 µl

The transfection mix was vortexed for 15 seconds and then incubated at room temperature for 15 minutes.

A volume of 1600 µl (6-well plate) or 400 µl (24-well plate) complete medium per well was added to the cells followed by addition of the transfection mix. Incubation of the transfected cells was started 30h after siRNA transfection.

2.3.2. DNA plasmids

SCC13 cells were transfected using Lipofectamine2000. Cells were seeded on 6-well plates or 12-well plates at 70% confluence. The transfection mix was prepared according to table 2.

Tab. 2: Preparation of transfection mix per reaction with Lipofectamine2000.

	6-well plate	12-well plate
OPTI-MEM	2x150 µl	2x75 µl
Lipofectamine2000	5 µl	2.5 µl

DNA	4 µg	2 µg

For each reaction, 5 (2.5) µl Lipofectamine2000 was added to 150 (75) µl OPTI-MEM and carefully mixed. 4 (2) µg DNA was added to another tube with 150 (75) µl OPTI-MEM. After 10 min incubation, the content of both tubes was combined and mixed by carefully pipetting up and down. In the meantime, cells were washed with PBS to remove FCS and 700 (350) µl OPTI-MEM was added to each well. The mix was incubated for 25 min at RT and then added drop-wise to the cells. After 4h the medium was changed to 2 ml DMEM/FCS.

Primary cells were transfected with Gene Carrier-1. Cells were seeded on 6-well plates or 12-well plates at 50% confluence. The transfection mix was prepared according to table 3.

Tab. 3: Preparation of transfection mix per reaction with Gene Carrier-1.

	6-well plate	12-well plate
Basal medium	2x100 µl	2x50 µl
Gene Carrier-1	4 µl	2 µl
DNA	1 µg	0.5 µg

For each reaction, 100 (50) µl basal medium were mixed with 4 (2) µl of Gene Carrier-1. In a separate tube, 100 (50) µl basal medium were mixed with 1 (0.5) µg DNA. Both mixes were then combined and incubated for 30 min at RT. In the meantime, cells were washed with basal medium and then 800 (400) µl basal medium were added per well. After the incubation, the mix was added drop-wise to the cells. After 5h, 1 (0.5) ml CnT07 medium was added to the wells.

2.4. Viability and proliferation assays

2.4.1. MTT assay

Cells for MTT assay were grown to 50% confluence and then treated with the drugs at indicated concentrations for 24h. Cells transfected with siRNA were treated 30h after transfection for 24h. MTT powder was dissolved in PBS to obtain a stock concentration of 5 mg/ml. After 24h incubation with the drugs, the MTT solution was added to each

well (end concentration: 250 µg/ml) and incubated for 1h at 37°C and 5% CO_2 in the dark. Afterwards, the MTT-containing medium was removed and equal amounts of Isopropanol+formic acid (Solution A) and SDS (Solution B) (100 µl for 96-well, 200 µl for 24-well) was added to the cells. After 5 min incubation at 37°C in the dark, absorbance was measured at a wavelength of 595 nm with background subtraction at 620 nM. For data analysis all measurements were normalized to DMSO treatment.

2.4.2. BrdU assay

Cells were seeded on 96-well plates at 50% confluence and treated with varying drug concentrations and for different durations as indicated. Additionally, BrdU stock solution was added to each well. After incubation, cells were fixed and the BrdU detection and measurement was performed according to the manufacturer's protocol. For data analysis all measurements were normalized to DMSO treatment.

2.5. Drug treatment

2.5.1. Cell culture

Cultured primary cells and cell lines were incubated with IM, TPA and/or indicated kinase inhibitors at varying concentrations. For cell culture treatment all drugs used were dissolved in DMSO, which served as control treatment. For treatments longer than 24h, IM was newly added to the cells every 24h. All cells were incubated at 37°C and 5% CO_2.

2.5.2. Skin explants

Skin explants were pre-incubated in CnT07 medium supplemented with either DMSO or PKC inhibitor for 2h before starting the actual IM treatment. After the pre-incubation, skin explants were placed on semi-solid culture medium and surrounded with either DMSO- or PKC inhibitor-containing medium. The epidermis of each explant was treated with either ingenol mebutate gel at indicated concentrations or with vehicle control. Skin explants were incubated at 37°C and 5% CO_2.

2.6. RNA extraction

After termination of the incubation, the medium was removed from the cells and TRIZOL® (Invitrogen) was added to the cells (1 ml TRIZOL per 6-well). For skin explants, epidermis and dermis were separated by a heat-shock (1 min in PBS at 60°C) followed by 1 min incubation in ice-cold 10% PBS and manual separation. The

epidermis was then transferred in 1 ml TRIZOL®. RNA isolation was performed according to the manufacturer's protocol. Afterwards, extracted RNA was purified by using the RNA clean up kit according to the manufacturer's manual (Qiagen). RNA quality and amount was then measured with the NanoDrop and considered to be of good quality when the 260/280 ratio and the 260/230 ratio were higher than 1.8.

2.7. Protein extraction

After termination of the incubation the medium was removed and cells were washed once with PBS followed by immediate incubation on ice. Depending on the amount of cells per well, 20-50 µl of ice-cold RIPA buffer containing protease inhibitors and phosphatase inhibitors were added to the cells. Lysed cells were scratched off the well with a cell scraper, transferred to a 1.5 ml Eppendorf tube and incubated on ice before being centrifuged at 12.000 rpm and 4°C for 15 min. The supernatant was then transferred to a new Eppendorf tube and either frozen at -20°C or further processed for SDS-PAGE. The protein concentration was measured by using the Dc protein assay kit (BioRad) according to the manufacturer's protocol.

2.8. Reverse transcription and qPCR

For generation of cDNA the GoScript Reverse Transcription Kit was used according to the manufacture's manual (Promega). Oligo(dT) primer and 1 µg of RNA were used for cDNA synthesis.

For quantitative PCR, a master mix for each gene of interest was prepared consisting of 10 µl FastStart Universal SYBR Green Master, 8 µl H_2O and 0.5 µl forward primer and 0.5 µl reverse primer per reaction. 19 µl of the master mix was added to each well of an optical 96-well reaction plate followed by addition of 1 µl cDNA per well. For each sample duplicates or triplicates on one plate were prepared. The plate was then sealed with a plastic cover and centrifuged to spin down the liquid and remove air bubbles. The qPCR was then performed on the ViiA7 real-time PCR machine with the following program: hold stage (95°C, 10 min), PCR stage (95°C, 10 sec; 60°C, 30 sec) repeat for 40 cycles, melt curve stage (95°C, 15 sec; 60°C, 1 min; 95°C, 15 sec).

Data were analyzed by calculating fold change of differential gene expression, normalized to the housekeeping gene 36B4.

2.9. SDS-PAGE, Western blotting and protein detection

2.9.1. SDS-PAGE

For further analysis, total protein extracts were size-separated by SDS polyacrylamid gel electrophoresis (PAGE). Therefore, 20 µg of total protein extract was mixed with 3x gel loading buffer (Laemmli buffer) and boiled for 5 min at 95°C. Protein samples were then loaded on an acrylamid gel, depending on the size of the protein of interest. For small proteins (up to 30 kDa) a 12% acrylamid gel was used, for proteins up to 80 kDa a 10% gel was used and for bigger proteins, an 8% gel was used. The gels were prepared according to table 4. During the run, the gel was placed in running buffer. The gel ran at a constant voltage of 120 V for approx. 90 min.

Tab. 4: Preparation of SDS gels (15 ml for 2 resolving gels, 6 ml for 2 stacking gels)

Components	8% resolving gel	10% resolving gel	12% resolving gel	5% stacking gel
H_2O	6.9	5.9	4.9	4.1
30% Acrylamide mix	4.0	5.0	6.0	1.0
1.5M Tris pH 8.8	3.8	3.8	3.8	
1M Tris pH 6.8				0.75
10% SDS	0.15	0.15	0.15	0.06
10% APS	0.15	0.15	0.15	0.06
TEMED	0.01	0.01	0.01	0.01

2.9.2. Western blotting

The proteins were then transferred from the gel onto a PVDF membrane by western blotting. Before transferring the proteins, the membrane was activated by pure methanol. The transfer was running in transfer buffer at constant voltage of 100 V for 1h45min. Afterwards, the membrane was incubated in blocking solution for 1h at room temperature on a shaker.

2.9.3. Protein detection

After blocking, the membrane was incubated in the primary antibody solution for the protein of interest. The primary antibodies were diluted in 5% milk as indicated in table 5.

Tab. 5: Dilution of primary antibodies for protein detection by western blotting.

Antibody	Dilution	Company
Anti-pPKCδ(Y311)	1:1000	Cell Signaling
Anti-pPKCδ(T505)	1:1000	Cell Signaling
Anti-PKCδ	1:1000	Cell Signaling
Anti-pERK	1:1000	Cell Signaling
Anti-ERK	1:1000	Cell Signaling
Anti-β-Actin	1:1000	Santa Cruz Biotechnology
Anti-CD32B		
Anti-RAGE	1:500	Millipore

The membranes were incubated over night at 4°C on a rotator. After incubation, the membranes were washed 3 times 10 min in TBS/T on a shaker and then incubated in the corresponding secondary antibody at the indicated concentration (table 6).

Tab. 6: Dilution of secondary antibodies for protein detection by western blotting.

Antibody	Dilution	Company
Anti-mouse HRP	1:10.000	Abcam
Anti-rabbit HRP	1:5.000	Cell Signaling
Anti-goat HRP	1:8.000	Santa Cruz Biotechnology

The membranes were incubated at RT for 1h followed by 3 times 10 min washing with TBS/T on a shaker.

For protein detection, ECL solution was used. The membranes were incubated in a mix of equal amounts of both solutions for 1 min at RT to elicit the chemiluminescent reaction that was afterwards captured by exposing the membrane to a film for varying length of time. The films were then developed in an automated developing machine.

After protein detection, the membranes were washed in TBS/T followed by incubation in stripping buffer for 15 min at RT on a shaker to remove the attached antibodies so that the membranes can be re-probed to detect other proteins. After stripping, the membranes were blocked again for 1h at RT on a shaker before they were incubated with another primary antibody.

As loading control for phospho-proteins, the amount of the total protein was used. As loading control for non-pohospho-proteins, β-actin was used as loading control.

2.10. Immunohistochemistry

For immunohistochemistry tissue was fixed in 4% formalin and paraffin-embedded. For staining 7 µm sections were deparaffinized by heating and xylen and then dehydrated. Antigen retrieval was achieved by incubation in EDTA. For detection of the specific proteins the same antibodies as for western blotting were used. Secondary antibodies as well as the staining kit were purchased from Dako. Afterwards, the sections were counterstained using haematoxylin.

2.11. Immunofluorescence staining and microscopy

For immunohistochemistry cells were seeded in 12-well plates or 6-well plates on cover slips. After finishing the experiment cells were fixed with 4% formalin for 20 min at RT. Afterwards cells were washed with PBS. For detection of proteins with fluorescent tags the cover slips containing the cells were directly attached to glass slides using ProLong® Gold Antifade Mountant containing DAPI for nuclear staining. Cells that needed to be stained for indirect immunofluorescence were washed with PBS and then permeabilized with PBS/0.1%TritonX100 for 15 min at RT. After washing the cells twice with PBS and once with PBS/0.1%Tween20 cells were blocked for 1h in PBS/1%BSA/0.1%Tween20 at RT. Cells were then washed twice with PBS/T and incubated with the primary antibody diluted in PBS/T for 1h at 4°C. After washing the cells twice in PBS/T cells were incubated with the secondary antibody diluted in PBS/T for 1h at 4°C in the dark. Cells were then washed twice with PBS/T and once with PBS. Then the cover slips were attached to the glass slides using ProLong® Gold Antifade Mountant containing DAPI for nuclear staining. Pictures were taken using the confocal laser scanning microscope SP5 from Leica.

2.12. Statistics

For statistical analysis of all data the software GraphPad Prism 5.0 was used. Mean and standard deviation were calculated out of at least three independent experiments. For comparison of two groups, unpaired Student's t-test with a confidence interval of 95% was used. For comparison of multiple groups, one-way ANOVA followed by Dunnet's multiple comparison was performed. For analysis of the tissue microarray the χ^2 was

used. Significant results are indicated by asterisks: *= $p<0.05$, **= $p< 0.01$, ***= $p< 0.001$, ****= $p< 0.0001$.

V. Results

1. Ingenol mebutate signals via PKC/MEK/ERK in keratinocytes and induces interleukin decoy receptors IL1R2 and IL13RA2

Freiberger, Sandra N.[1]; Cheng, Phil F.[1]; Iotzova-Weiss, Guergana[1]; Neu, Johannes[1]; Liu, Qinxiu[1]; Dziunycz, Piotr[1]; Zibert, John R.[2]; Dummer, Reinhard[1]; Skak, Kresten[2]; Levesque, Mitchell P.[1]; Hofbauer, Günther F.L.[1]*

[1] Department of Dermatology, University Hospital Zurich, Switzerland
[2] LEO Pharma A/S, Ballerup, Denmark

* Corresponding author: Günther F. L. Hofbauer, Department of Dermatology, University Hospital Zurich, Gloriastrasse 31, 8091 Zurich, Switzerland. Phone: +41 44 2551111; E-mail: Hofbauer@usz.ch

Running title: Ingenol mebutate's mechanism in keratinocytes

Keywords: cutaneous squamous cell carcinoma, ingenol mebutate, PKC, MAPK, interleukin decoy receptors

Financial support: LEO Pharma A/S supported this study with a research grant to Günther Hofbauer.

Disclosure of potential conflict of interest: Kresten Skak and John Zibert are employees of LEO Pharma A/S.

Word count: 4587

Number of figures: 5 figures and 6 supplementary figures

Number of tables: 1 supplementary table

1.1. Abstract

Cutaneous squamous cell carcinoma (SCC) is the second most common human cancer and the second leading cause of skin cancer-related death. Recently, a new compound, ingenol mebutate (IM), was approved for treatment of actinic keratosis (AK), a precursor of SCC. Since the mechanism of action is poorly understood, we have further investigated the mechanism of IM-induced cell death.

We elucidate direct effects of IM on primary keratinocytes, patient-derived SCC cells and on a SCC cell line. Transcriptional profiling followed by pathway analysis was performed on IM-treated primary keratinocytes and patient-derived SCC cells to find key mediators and identify the mechanism of action. Activation of the resulting pathways was confirmed in cells and human skin explants and supported by a phosphorylation screen of treated primary cells. The necessity of these pathways was demonstrated by inhibition of certain pathway components.

IM inhibited viability and proliferation of all keratinocyte-derived cells in a biphasic manner. Transcriptional profiling identified the involvement of PKC/MEK/ERK signaling in the mechanism of action and inhibition of this signaling pathway rescued IM-induced cell death after treatment with 100 nM IM, the optimal concentration for the first peak of response. We found the interleukin decoy receptors IL1R2 and IL13RA2 induced by IM in a PKC/MEK/ERK-dependent manner. Furthermore, siRNA knock-down of IL1R2 and IL13RA2 partially rescued IM-treated cells.

In conclusion, we have shown that IM-induced cell death is mediated through the PKCδ/MEK/ERK pathway, and we have functionally linked the downstream induction of IL1R2 and IL13RA2 expression to the reduced viability of IM-treated cells.

1.2. Introduction

Actinic keratoses (AKs) are hyperkeratotic lesions on sun-exposed surfaces such as the face, scalp and lower arms. AKs are caused by accumulated UV exposure over lifetime and develop from atypically proliferating keratinocytes (1). In about 8% of cases, AKs can progress to invasive SCCs (2), the second most common form of skin cancer, and therefore need to be treated. About 50% of SCCs harbor p53 mutations, some of them with the typical UV signature of cyclobutane pyrimidine dimers (3). The risk for organ transplant recipients to develop SCC is highly increased due to their treatment with

immunosuppressive drugs (4) such as azathioprine, a photosensitizer to UVA light (5) or cyclosporine A inducing the protumorigenic transcription factor ATF3 (6, 7). The incidence of SCC in the general population continues to increase with high morbidity and low mortality (8). In contrast to basal cell carcinoma (BCC), SCC carries a risk of metastasis. The current treatment options for actinic keratosis range from topical treatments with gels or creams for field cancerization to cryotherapy for single lesions (9), whereas SCC is normally treated by surgical excision. Self-directed treatments are preferred by patients, but are time consuming as they need to be applied for weeks or months to produce clinical results.

Recently a new compound, ingenol mebutate (IM), was registered in the USA and Europe for topical treatment of AK in two different concentrations depending on the treatment location. IM showed 42.2% complete clinical clearance compared to placebo (10) and has the great advantage of a short treatment duration (i.e. two to three consecutive days), a short period of local skin reaction and no systemic adverse events (11, 12). Although IM is registered and used in the clinic, its exact mechanism of action is not fully understood. Recent studies suggest a dual mechanism of action with rapid initial necrosis (13) followed by activation of the innate immune system (14). Although several reports investigated the mechanism of action of IM on different cancer cell lines like colon cancer (15), melanoma (16) and leukemia (17), there is only one report studying the effect on epithelial cells (18). In that paper, the authors demonstrate acute cytotoxicity of clinical drug concentrations on cancer cell lines and keratinocytes with disruption of the mitochondrial network and the involvement of intracellular calcium release.

To better characterize the mechanism of action of IM in epithelial cells, we analyzed the impact of IM on the proliferation and viability of primary keratinocytes, primary patient-derived SCC cells and a SCC cell line. Furthermore, we identified signaling pathways essential for IM action by gene expression analysis and confirmed their contribution in primary cell cultures and human skin explants. We functionally demonstrated the critical importance of several novel genes using functional in vitro assays.

1.3. Results

Ingenol mebutate inhibits viability and proliferation of keratinocytes and SCC cells

To investigate the impact of IM on epithelial cell viability and proliferation and to determine a working concentration for further experiments, we treated primary keratinocytes, patient-derived SCC cells, and SCC cell lines for 24h, 48h and 72h with IM in a range from 1 nM to 105 nM. All cell types showed a biphasic decrease in viability (Fig. 1A) and proliferation (Fig. 1B) upon drug treatment. Interestingly, all cells exhibited a drop in viability and proliferation around a concentration of 100 nM, while cells appeared healthier at lower (1-10 nM) and higher (103-104 nM) concentrations (Fig. 1A and 1B). At a concentration of 105 nM, cells died to a great extent due to cytotoxicity. For further experiments we used a concentration of 100 nM, since this concentration had the largest effect at the first peak of the biphasic response.

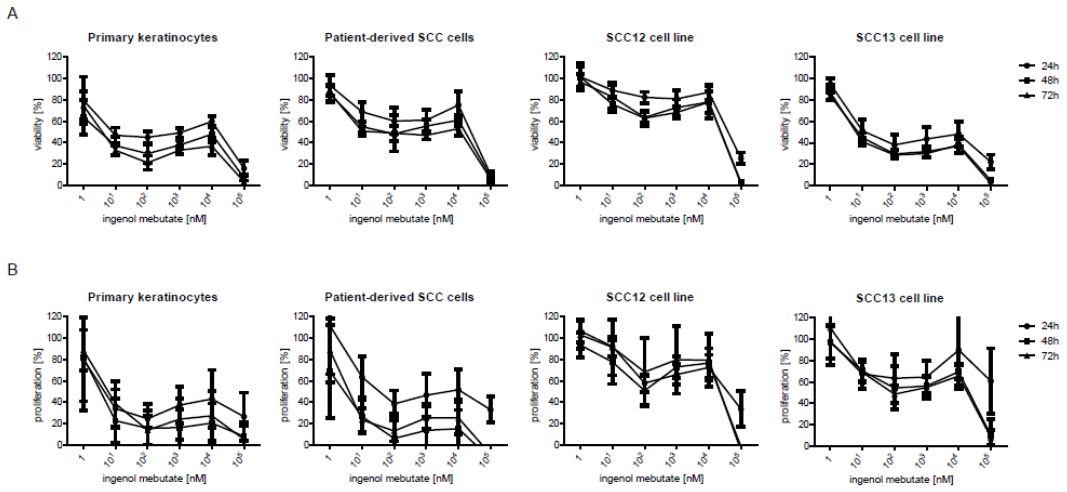


Figure 1. Ingenol mebutate affects viability and proliferation of keratinocytes and SCC cells. Primary keratinocytes and patient-derived SCC cells of three different donors and the human squamous cell carcinoma cell lines SCC12 and SCC13 were treated with the indicated concentrations of ingenol mebutate for 24h, 48h or 72h. A, cell viability measured by MTT assay B, cell proliferation measured by BrdU incorporation. All values were normalized to DMSO-treated cells. Graphs represent mean and standard deviation of three independent experiments.

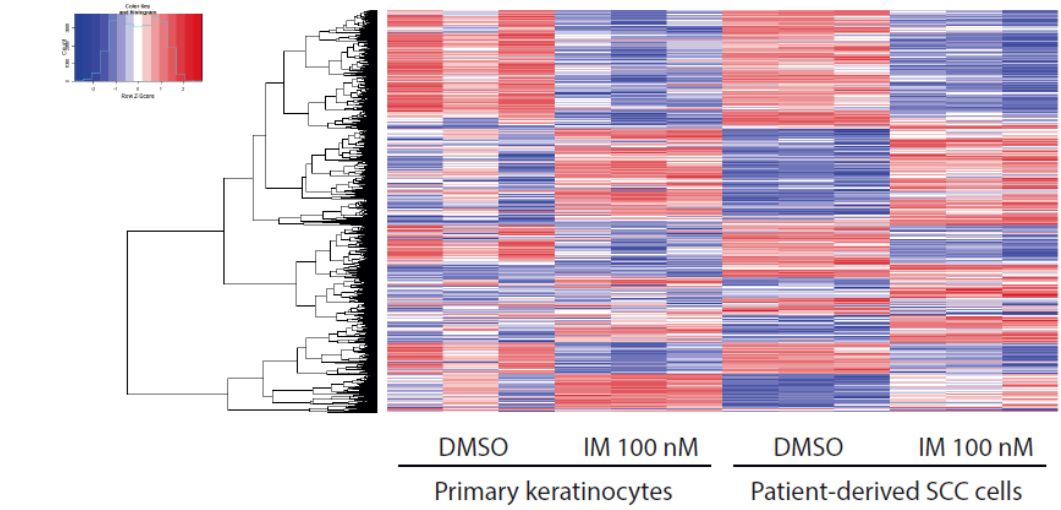
To find out whether the biphasic effect was cell-type specific, we additionally treated fibroblasts and melanoma cells with similar concentrations of IM for 24h. We found no biphasic effect in fibroblasts, since they were resistant to the highest concentration used (105 nM). Melanoma cells did not show a biphasic effect either, since they were almost unaffected at low concentrations, whereas viability was 50% decreased at the highest concentration used in the experiment (105 nM) (Suppl. Fig. 2A). Furthermore, we

analyzed the viability of the SCC13 cell line after 24h treatment with several concentrations of the tumor promoting tetradecanoyl phorbol acetate (TPA), a drug structurally related to the anti-cancer drug IM, both being phorbol esters and known potent PKC activators (22). This was done to investigate if the effect observed with IM was drug- specific. Unlike IM we did not detect any biphasic effect upon TPA treatment (Suppl. Fig. 2B).

Gene expression analysis reveals involvement of PKC and MAPK signaling

To identify genes and signaling pathways mediating IM activity, we performed gene expression analysis on primary keratinocytes and patient-derived SCC cells treated for 24h with either DMSO or different concentrations of IM (1 nM, 100 nM, 104 nM). In concordance with the results from the viability and proliferation assays, most genes were differentially expressed in the presence of 100 nM IM (Fig. 2A). Fewer changes were detected at a concentration of 104 nM while 1 nM of IM had almost no effect (Suppl. Fig. 2A). At a concentration of 100 nM, we found 1227 genes upregulated in keratinocytes and 795 in SCC cells. 390 of these genes overlap between the two cell types. Furthermore, we found 1887 downregulated genes in keratinocytes and 996 in SCC cells with 713 genes overlapping (Fig. 2B).

A



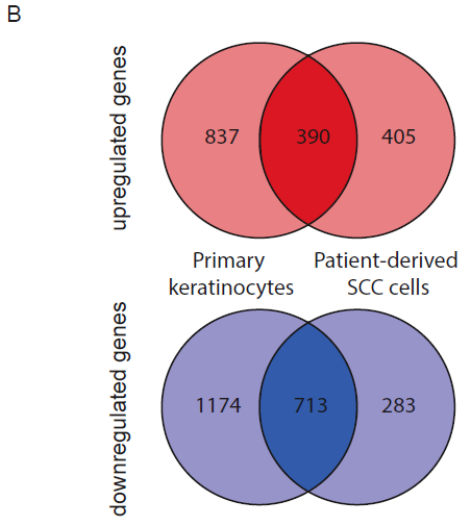


Figure 2. Gene expression analysis of IM-treated primary keratinocytes and patient-derived SCC cells. A, Heatmap showing triplicates of differentially expressed genes between DMSO-treated and IM-treated primary keratinocytes and patient-derived SCC cells. Cells were treated with either DMSO or 100 nM IM for 24h followed by RNA extraction and performance of the ¬microarray. B, Number of differentially expressed genes in primary keratinocytes and patient-derived SCC cells after treatment with 100 nM IM. Upregulated genes are displayed in red, downregulated genes are displayed in blue.

Hierarchical clustering of the microarray data showed common clusters of gene expression patterns in the keratinocytes and SCC cells upon drug treatment, as compared to vehicle-treated cells. Pathway analysis of down-regulated genes using the software GeneGo identified mostly cell cycle-associated pathways and DNA damage repair pathways (Suppl. Fig. 3B). Upregulated genes following 100 nM IM treatment mostly affected pathways such as ERK1/2- and Protein Kinase C (PKC)-signaling in keratinocytes and ERK1/2 signaling in SCC cells (Suppl. Fig. 3C).

To identify key mediators of the IM effect, we took the top 5 upregulated genes in SCC cells that were also upregulated in keratinocytes. DEFB4A, ZP4, IL13RA2, CCL5, and IL1R2 were thus selected. We did not focus on C15orf48, which was also was among the top 5 upregulated genes, since we were interested in genes that are upregulated in both healthy and cancerous keratinocytes and C15orf48 was only found to be upregulated in SCC cells. Furthermore, we could not confirm the upregulation of C15orf48 in SCC tissue (data not shown). From literature research, we found DEFB4A, IL1R2 and IL13RA2 to be the most interesting ones. IL1R2 and IL13RA2 are decoy receptors that were thought to have no signaling function. However, a recent paper demonstrated a signaling ability of IL13RA2 (23). Additionally, IL13AR2 was reported to slow down or even prevent tumor

growth in mice when overexpressed in pancreatic or breast cancer cells (24). Furthermore, IL1β was shown to enhance proliferation of oral keratinocytes (25) and therefore the upregulation of its decoy receptor by IM may play a crucial role in the mechanism of action of IM. DEFB4A was shown to be differentially expressed in SCC (26). We then confirmed the upregulation of these genes by qPCR in keratinocytes, patient-derived SCC cells, two SCC cell lines, human epidermis and human SCC explants after 24h of IM treatment. We found IL1R2 and IL13RA2 consistently and significantly upregulated in all tested cell types and explant tissues after treatment with IM, while DEFB4A upregulation could not be confirmed in SCC cell lines and SCC explants (Suppl. Fig. 3D, suppl. tab. 1).

PKCδ plays a fundamental role in the mechanism of action of ingenol mebutate

As our pathway analysis agreed with earlier studies (16, 22) that indicated a role for PKC signaling following IM exposure, we further investigated this pathway as a possible mechanism of action in the loss of epithelial cell viability and proliferation. We incubated primary keratinocytes, patient-derived SCC cells and the SCC13 cell line with 100 nM IM for up to 45 minutes and found PKCδ to be highly phosphorylated in the hinge region (Y311) in all cell types while PKCα is not phosphorylated (Fig. 3A). Y311 was previously reported to be required for PKCδ activation and furthermore enhances autophosphorylation of PKCδ on T505 (27). Accordingly, we found the activation loop (T505) of PKCδ to be more phosphorylated in all three cell types after 24h drug treatment, whereas this phosphorylation could be blocked by the PKC inhibitor AEB071 (Fig. 3B). Although PKCδ can function without being phosphorylated at T505, this phosphorylation increases the catalytic activity of PKCδ (27). Furthermore, T505 phosphorylation influences the substrate specifity of PCKδ and is essential for the activation of the AP1 family of transcription factors (28).

To further investigate the role of PKCδ, we used IM alone or in combination with the PKC inhibitor AEB071 and measured cell viability of primary keratinocytes, SCC patient cells and the SCC13 cell line after 24h of treatment. We found cell viability to be partially rescued by PKC inhibition when treated with IM (Fig. 3C). Since the PKC inhibitor is not entirely specific for single isoforms, we further validated the role of PKCδ using siRNA to knock-down PKCδ in SCC13 cells followed by analysis of cell viability after 24h IM

treatment. Similar to our findings with the PKC inhibitor, we detected a rescue of cell viability upon IM treatment when PKCδ was knocked down. A knock-down PKCα followed by IM treatment led only to a partial rescue, while PKCδ knock-down could rescue the IM-treated cells completely (Fig. 3D).

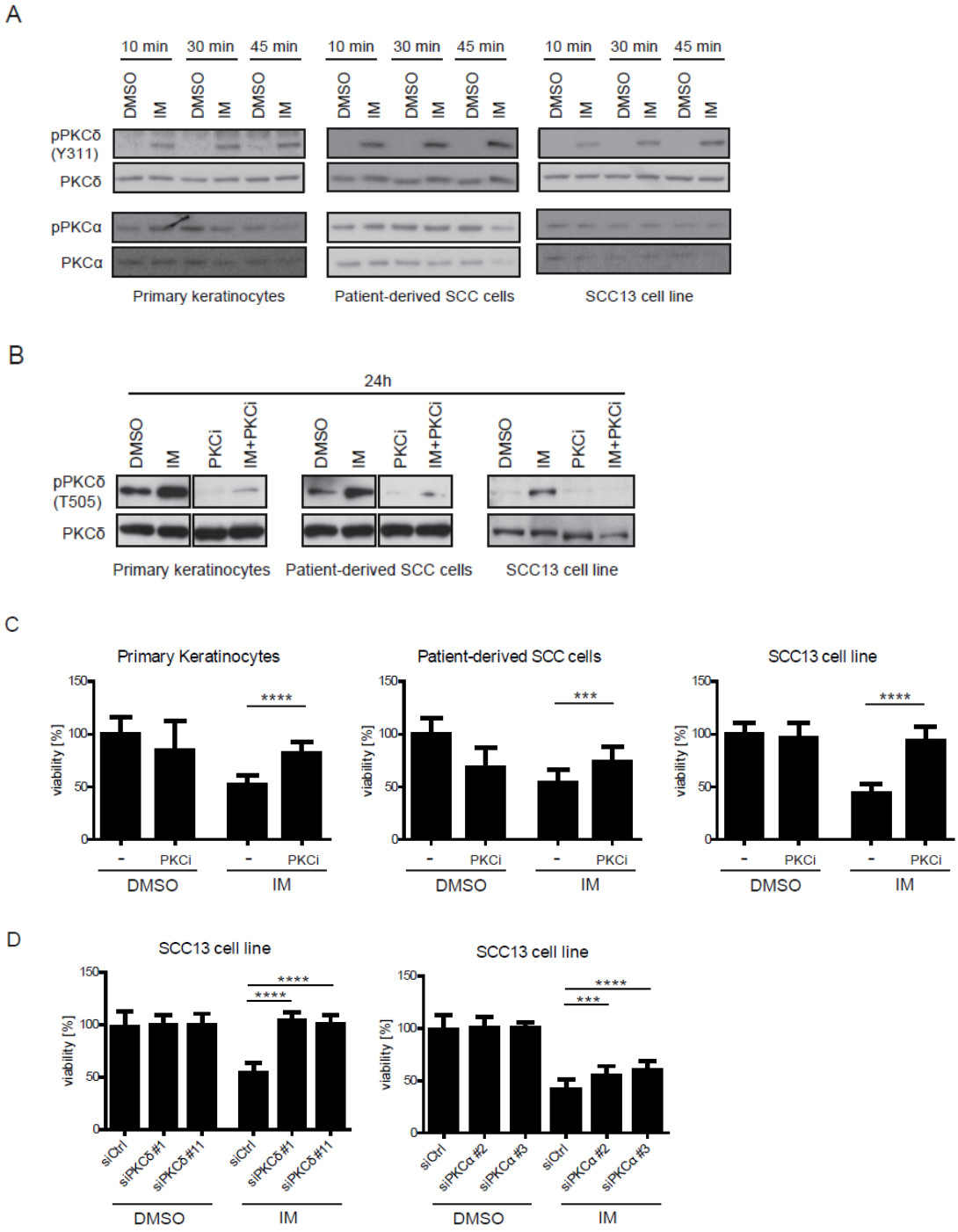


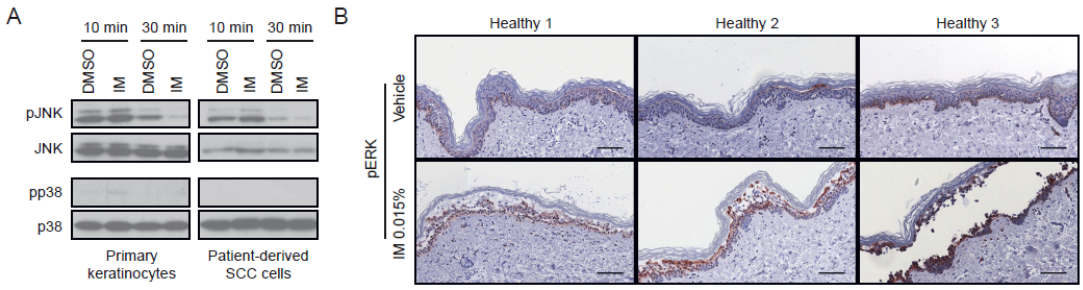
Figure 3. PKCδ is phosphorylated after IM treatment and is essential for the performance of IM. A, Primary keratinocytes and SCC13 cells were treated with 100 nM IM for 10 min, 30 min or 45 min followed by

protein extraction. Phosphorylation of PKCδ Y311 and PKCα was detected by western blotting. Total PKCδ or PKCα was used as loading control. B, Primary keratinocytes, patient-derived SCC cells and SCC13 cells were treated for 24h with 100 nM IM alone or in combination with the PKC inhibitor AEB071 followed by protein extraction. Phosphorylation of PKCδ T505 was detected by western blotting. Total PKCδ was used as loading control. C, Primary keratinocytes, patient-derived SCC cells and SCC13 cells were treated for 24h with 100 nM IM alone or in combination with the PKC inhibitor AEB071 followed by assessing cell viability by MTT assay. Graphs represent mean and sd of three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnet's multiple-comparison test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. D, SCC13 cells were transfected with two different siRNA against PKCδ (left panel) or PKCα (right panel) followed by 24h treatment with 100 nM IM and followed by assessing cell viability by MTT assay. Graphs represent mean and SD of three independent experiments. Data were analyzed by Student's t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

ERK is activated by ingenol mebutate treatment in a PKC-dependent manner and mediates IM-dependent effects on cell viability

Besides PKC signaling, our pathway analysis suggested ERK1/2 to be involved in mediating the effect of IM. To confirm this and to exclude other pathways involved in MAPK signaling (e.g. p38, JNK) we performed a phosphorylation array experiment with primary keratinocytes and SCC cells from three different donors and found ERK to be phosphorylated upon IM treatment, while other pathways of MAPK signaling (p38 and JNK) showed no or only weak phosphorylation (Suppl. Fig. 5A). Weak or nonexistent phosphorylation of JNK and p38 was further validated on cells from more donors by conventional western blotting (Fig. 4A). In order to test the effect of IM in a more natural model of skin, we treated human skin explants in three independent experiments with either vehicle or IM gel 0.05%. Assessment of ERK phosphorylation after 24h of treatment revealed a sustained activation of ERK compared to vehicle-treated samples (Fig. 4B). To further demonstrate activation of ERK, we analyzed the expression of known ERK target genes (EGR1, SPRY2, CFOS) (29) in SCC13 cells, primary keratinocytes and patient-derived SCC cells after 24h of treatment by qPCR and found them to be upregulated upon IM treatment, while the upregulation of the ERK target genes was abolished by an ERK inhibitor (SCH772984) and a MEK inhibitor (GSK1120212B) (Fig. 4C). Additionally, we used the PKC inhibitor AEB071 either alone or in combination with IM and analyzed the phosphorylation of ERK by western blotting after short-term treatment and the response of the ERK target genes after 24h IM treatment (Fig. 4D). The drug alone induced phosphorylation of ERK and ERK target

gene upregulation as shown in the previous experiment, which was blocked by addition of the PKC inhibitor. Furthermore, upregulation of ERK target genes after IM treatment was abolished by knock-down of PKCδ (Fig. 4D). This was confirmed in human skin explants pre-incubated in either DMSO or PKC inhibitor for 2h and treated with either vehicle or IM gel 0.015%. After 18h of incubation we analyzed pERK status by immunohistochemistry and found sustained ERK phosphorylation after drug treatment, while this phosphorylation was abolished by the PKC inhibitor (Fig. 4E). Taken together these results indicate that ERK is rapidly phosphorylated by IM in a PKC-dependent manner upon drug treatment. To further investigate these pathways in the mechanism of action of IM we analyzed the viability of primary keratinocytes, patient-derived SCC cells and the SCC13 cell line after 24h treatment with either the drug alone or in combination with the ERK inhibitor (SCH772984) or the MEK inhibitor (GSK1120212B). As seen in previous experiments, cell viability dropped below 50% upon IM treatment (Fig. 4F). However, viability was partially rescued when cells were treated in combination with the MEK or ERK inhibitor but not in combination with the JNK inhibitor SP600125 or the p38 inhibitor SB203580 (Fig. 4F).



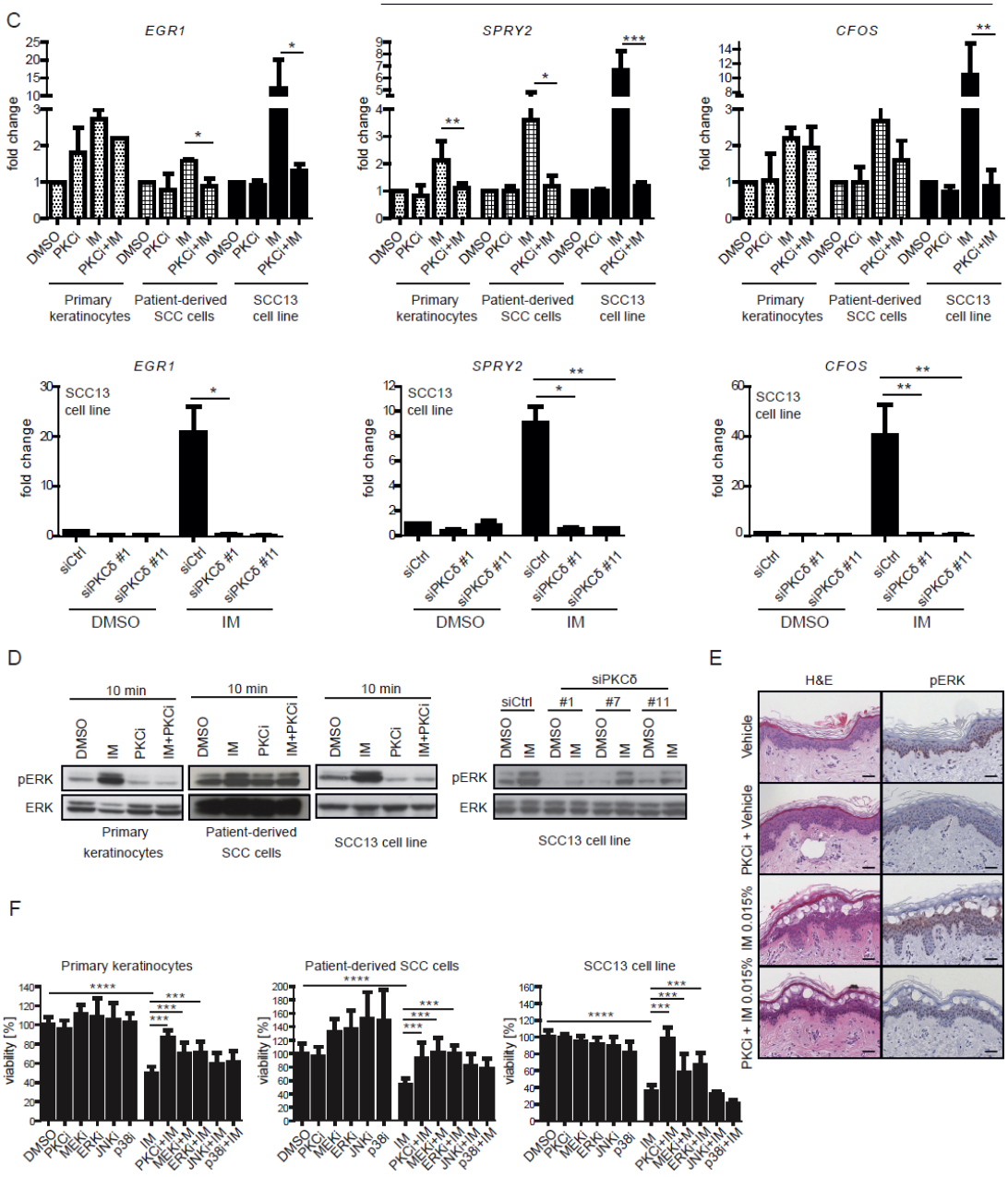


Figure 4. ERK is activated by IM treatment in a PKC-dependent manner. A, Phosphorylation of JNK and p38 was analyzed in total protein extracts of primary keratinocytes and patient-derived SCC cells after 10 min and 30 min of IM treatment. B, Immunohistochemical staining for ERK phosphorylation after 24h treatment with either vehicle or IM 0.05% in healthy skin organ cultures from three different donors. Scale bar = 100 µm. C, ERK target genes are upregulated after 24h of IM treatment and can be blocked by PKC inhibition or by knockdown of PKCδ. Gene expression was assessed by qPCR. Graphs represent mean and SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnet's multiple-comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. D, Phosphorylation of ERK after IM treatment can be blocked by the PKC inhibitor or by knockdown of PKCδ. Primary keratinocytes, patient-derived SCC cells and SCC13 cells were treated for 10 min with IM alone or in

combination with the PKC inhibitor followed by protein extraction. Moreover, SCC13 cells were transfected with two different siRNAs against PKCδ followed by 10 min IM treatment. Extracts were analyzed by western blotting and stained for ERK phosphorylation. Total ERK protein was used as loading control. E, Immunohistochemical staining of healthy skin for ERK phosphorylation after 18h treatment with either vehicle or IM 0.015% alone or in combination with the PKC inhibitor. Pictures show representative staining. Left panel: H&E staining, right panel: pERK staining, scale bar = 100 µm. F, Inhibition of PKC, MEK or ERK but not JNK or p38 partially rescues viability of IM treated cells. Primary keratinocytes, patient-derived SCC cells and SCC13 cells were treated for 24h with either IM alone or in combination with the PKC, MEK, ERK, JNK or p38 inhibitor. Viability was assessed by MTT assay. Graphs show mean and SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

IL1R2 and IL13RA2 partially mediate the mechanism of action of ingenol mebutate in SCC

To further investigate the role of IL1R2 and IL13RA2, we first confirmed their upregulation upon 24h IM treatment in keratinocytes, patient-derived SCC cells, the SCC12 and SCC13 cell lines and epidermis from human explants by qPCR (Suppl. Fig. 2D). C15orf48 was not further investigated due to the lack of a commercially available antibody and the fact that we could not confirm its upregulation in SCC tissue. Since we saw an upregulation of IL1R2 and IL13RA2 after 24h of treatment, we performed time course experiments in primary keratinocytes and epidermis from human skin explants to better define the timescale of gene regulation. We found both genes to be upregulated after 2-6h of treatment (Suppl. Fig. 4).

Furthermore we treated cells with IM alone or in combination with the PKC-, the MEK- or the ERK inhibitor respectively and found that the drug-dependent upregulation of both genes was partially abolished. IM in combination with JNK inhibition or p38 inhibition had almost no repressive effect on the expression of the two genes (Fig. 5A).

Additionally we knocked down PKC by siRNA, which also prevented the drug-dependent upregulation of IL1R2 and IL13RA2 mRNA (Fig. 5B).

To find out whether IL1R2 or IL13RA2 play an essential role in the mechanism of action IM, we knocked down these two genes with siRNA and analyzed cell viability upon drug treatment. Interestingly, both IL1R2 and IL13RA2 knock down partially rescued the viability of drug-treated SCC13 cells (Fig. 5C).

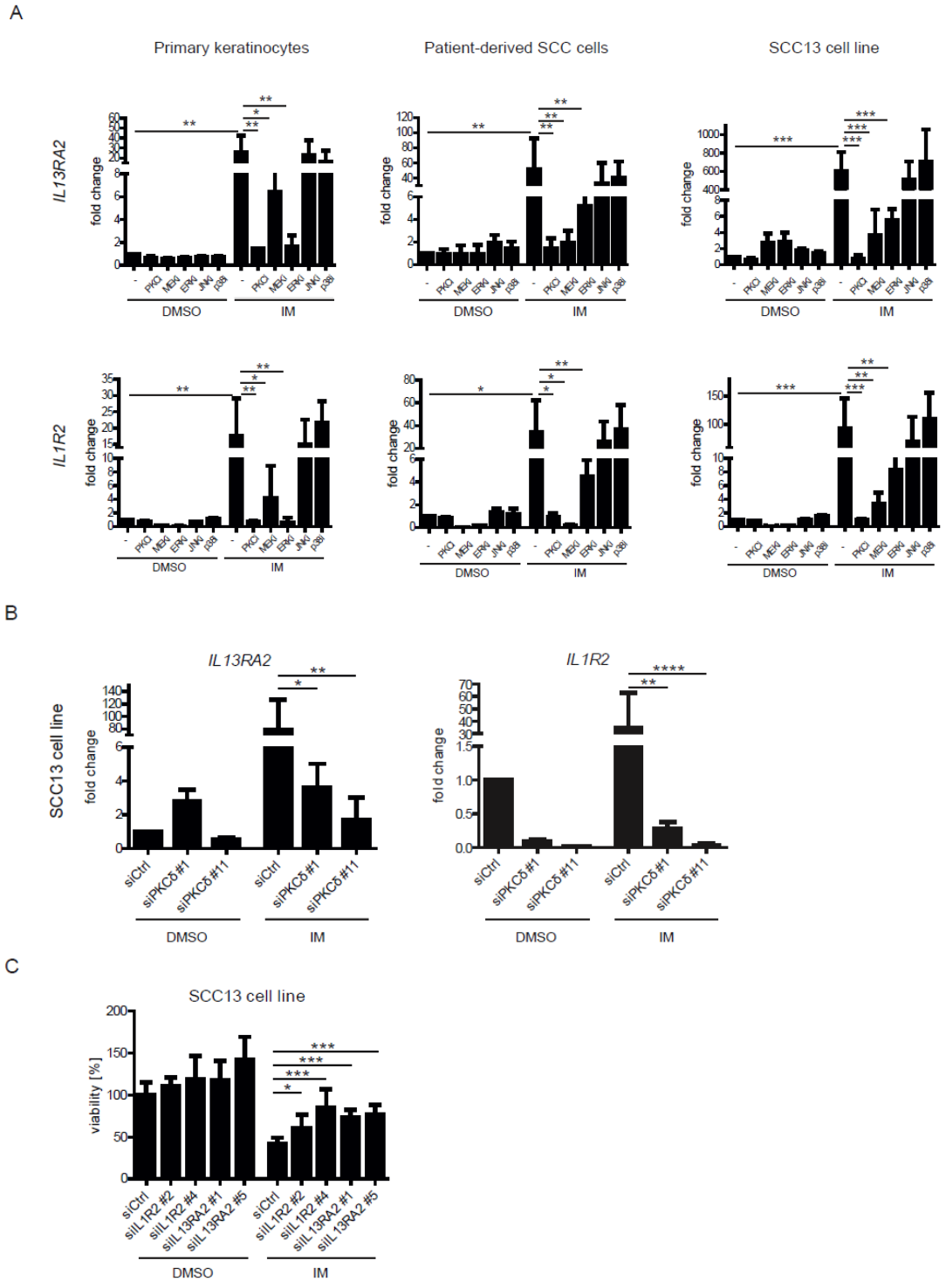


Figure 5. IL13RA2 and IL1R2 are involved in the mechanism of action of IM. A, *IL13RA2* and *IL1R2* gene expression is upregulated by IM treatment in a PKC/MEK/ERK -dependent manner. Cells were treated either with IM alone or in combination with the indicated kinase inhibitors for 24h followed by RNA isolation and gene expression analysis by qPCR. Data of at least three independent experiments were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. * = p<0.05, ** = p<0.01, ***

= p<0.001, **** = p<0.0001 . B, IL13RA2 and IL1R2 are PKCδ-dependent. SCC13 cells were transfected with siRNA against *PKCδ* and treated for 24h with 100 nM IM. Cell viability was assessed by MTT assay. C, Knock-down of *IL13AR2* and *IL1R2* partially rescues viability of IM treated cells. SCC13 cells were transfected with two different siRNA against *IL13RA2* or *IL1R2* followed by 24h of IM treatment. Viability was assessed by MTT assay. All graphs (B, C) show mean and SD of three independent experiments. Data were analyzed by Student's t-test. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

1.4. Discussion

Ingenol mebutate (IM) is approved for the topical treatment of actinic keratosis (AK), the precursor of squamous cell carcinoma (SCC). However, its mechanism of action is not completely understood. Several studies investigated IM in the context of different cancers (15-17) and suggest a role of the innate immune system (14) but there is only one study that focuses on the effect on the actual target, the malignant keratinocytes (18). While that study used high IM concentrations (100-400 µM) and short incubation times, leading to cytotoxicity involving mitochondrial network disruption, we investigated the mechanism of action of IM in healthy keratinocytes, SCC cells and skin explants at lower concentrations (100 nM) and up to 72h. We showed that IM induced a direct effect on viability and proliferation on cells of different origin. Previous studies on different mouse and human cancer cell lines, like melanoma or breast carcinoma, determined the lethal dose (LD90) of IM for these cells to be between 180 and 220 µM after 24h (13). Benhadji et al. assessed growth inhibition by MTT assay and calculated the IC50 of colon cancer cell lines, finding great variations from 3 µM in Colo-205 cells to more than 300 µM in HCT116 and HCC2998 cells (30). Overall the concentrations leading to cell death strongly vary depending on the cell line tested and our results fit within this range showing most cells reaching a lethal affect at 100 µM IM. In line with other reports (16), we found fibroblasts to be relatively resistant to IM while melanoma cells were affected, but at higher concentrations than the keratinocytes. In contrast to melanoma cells and fibroblasts, we found a biphasic effect on viability and proliferation on keratinocytes and SCC cells. This determines that IM induces cell death in two ways, depending on concentration and cell type. Furthermore, our results on SCC13 cells indicated that the biphasic effect was IM-specific as compared to another PKC activator, TPA. Our microarray performed with various IM concentrations showed the highest number of genes differentially expressed at 100 nM, whereas effects were less pronounced at lower and higher concentrations, consistent with the impact of 100 nM IM on cell viability and proliferation. This pronounced effect at 100 nM again points to

the existence of two different concentration-dependent mechanisms of action for IM, whereas the highest concentration used leads to cell death due to direct cytotoxicity. The lower concentration of 100 nM however, may trigger intracellular pathways that lead to the reduction of cell viability. These potential signaling pathways were subject to our further investigations.

PKCδ is a key mediator in cell differentiation and inhibition of proliferation in various tissues (31-34). A direct binding of IM to PKC isoforms has been observed (22). IM treatment leads to phosphorylation of PKCδ in the Colo-205 cancer cell line (30). In other studies, PKCδ was reported to drive keratinocyte differentiation and inhibit proliferation of the immortalized keratinocyte line HaCaT, which could be reversed by PKCδ inhibition (32). Our data from gene expression to functional experiments in primary cell cultures underline a critical role for PKCδ in mediating the effect of IM in keratinocytes and verify such previous observations correspondingly for IM.

Downstream of PKC signaling, our pathway analysis revealed an involvement of ERK1/2 in the mechanism of action of IM. Previous work on colon cancer cells suggests involvement of MAPK pathways including JNK and p38 (30, 35) by showing phosphorylation of these proteins after IM treatment. We confirmed activation of ERK1/2 while we were able to exclude an involvement of other MAPK pathway components like JNK and p38 in keratinocytes and SCC cells. Similar to previous studies on melanoma cells (16), our data on keratinocytes clearly link PKCδ to the activation of MAPK signaling. Both MEK and ERK are essential for the effect of IM in our assays. Although the activation of the MEK/ERK pathway is generally considered to promote tumor cell growth (36), ERK activation seems to contribute favorably to the mechanism of action of IM, leading to reduced cell viability and proliferation. In line with this assumption, ERK activation was found to suppress tumors by promotion of selective protein degradation (37). Moreover, high ERK activation can serve as a marker of improved outcome in breast cancer patients (38).

We identified and validated a role for several novel genes as critical players in the mechanism of action of IM. Our analysis focused on IL13RA2 and IL1R2, which were both induced by IM in a PKCδ/MEK/ERK-dependent manner. Both genes encode for

interleukin receptors that are commonly known to function as decoy receptors without signaling ability (39, 40). Nevertheless, a recent publication attributed an active role to IL13RA2 in signaling, thus inducing TGFβ-mediated fibrosis (23). However, to the case for IM induced effects, induction of fibrosis is unlikely as patients treated with IM do not show (fibrosis-related) scarring and have a good cosmetic outcome post-treatment with IM gel (11). Moreover, a recent study revealed a role of IL13RA2 in tumor suppression, as IL13RA2-overexpressing breast and pancreatic cancer cells showed reduced or no tumor growth when injected into mice (24).
Similarly, IL1R2 recently showed activity against ectopic tissue growth and endometriosis progression and could down-regulate the anti-apoptotic protein Bcl2 (41), which could explain that the cell death mechanism by IM could be attributed by IL1R2 signaling. As IL1β was shown to promote proliferation of oral keratinocytes (25), one could speculate whether IL1R2 is upregulated by IM to capture IL1β and prevent this effect. Further studies are needed to clarify the role of IL13RA2 and IL1R2 in the mechanism of action of IM.

In summary, we report a mechanism of action for IM in proliferating normal and malignant keratinocytes through specific activation of PKCδ leading to activation of MEK/ERK signaling, resulting in decreased viability. This was dependent on concentration and cell type, resulting in a unique biphasic induced loss of viability through IM. A set of genes responsive to IM treatment, mainly IL13RA2 and IL1R2, showed that they partially mediate the function of IM on SCC, suggesting a function for them apart from their role in the immune system. However, our data do not allow us to conclude whether IL13RA2 and IL1R2 exert their effect primarily in keratinocytes treated by IM or whether they are instrumental in orchestrating the accompanying immune response. Further projects are needed to shed more light on this matter.

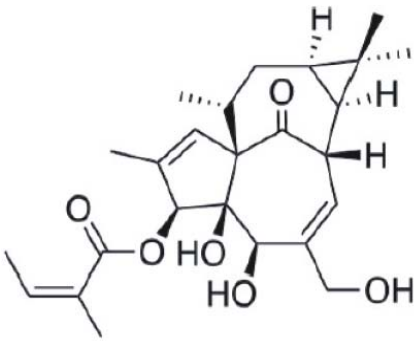
1.5. Acknowledgements

The gene expression array was done by the Functional Genomic Center Zurich (FGCZ). The authors thank Ines Kleiber-Schaaf (Dept. of Dermatology, University Hospital Zurich) for immunohistochemical staining. The University Research Priority Program in translational cancer research at the University of Zurich provided melanoma and fibroblast cultures used here.

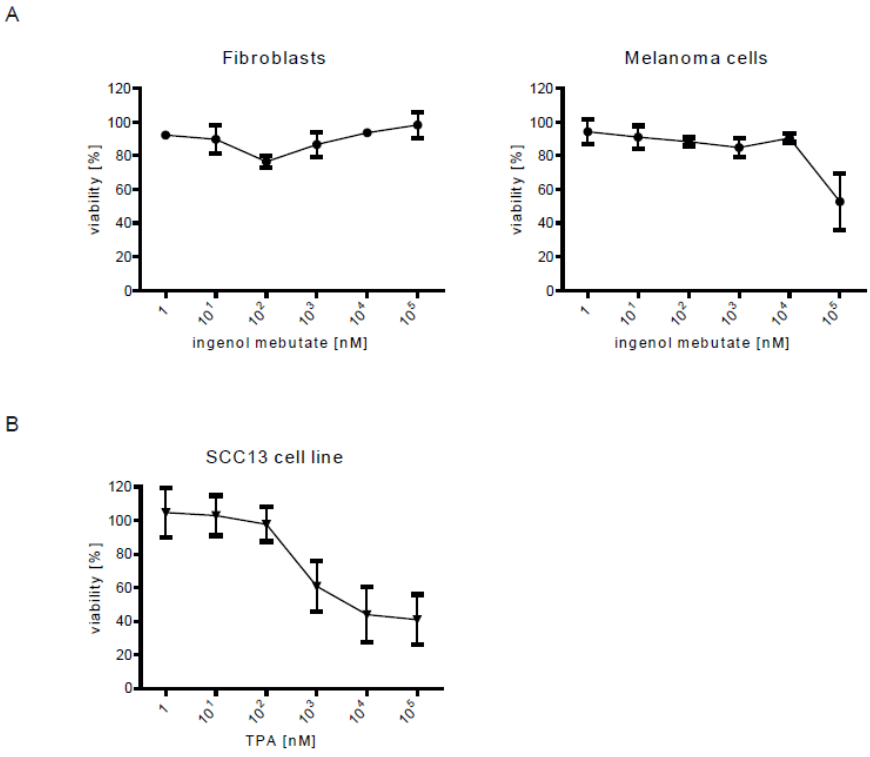
1.6. Supplementary data

Supplementary Table 1. Selection of further investigated targets.

Patient-derived SCC cells				Primary keratinocytes	literatur research	qPCR
GENE_SYMBOL	**GENE_NAME**	**logFC**	**adj.P.Val**	position in PK		
DEFB4A	defensin; beta 4A	7.487065906	0.001829842	1	yes	not expressed in SCC13
C15orf48	chromosome 15 open reading frame 48	7.084376309	0.002246738	NA		
ZP4	zona pellucida glycoprotein 4	6.913020027	4.38E-05	14	no	
IL13RA2	interleukin 13 receptor; alpha 2	6.254209761	0.000454344	26	yes	yes
CCL5	chemokine (C-C motif) ligand 5	5.953273034	0.000839119	16	no	
IL1R2	interleukin 1 receptor; type II	5.69845889	6.90E-05	72	yes	yes



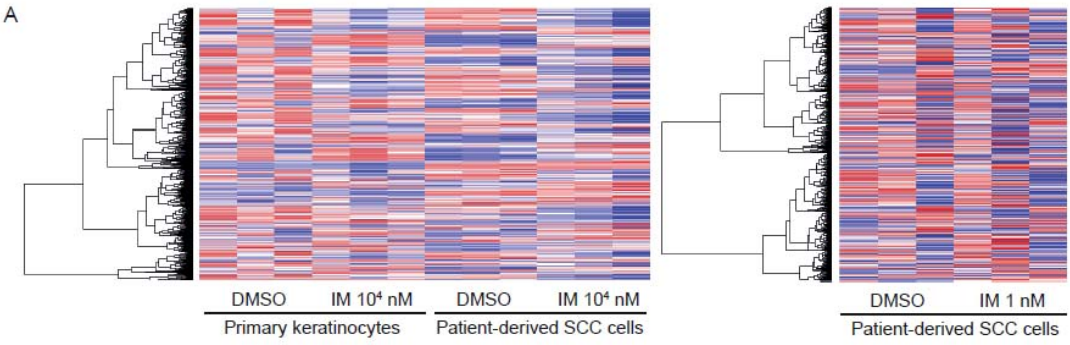
Supplementary Figure S1. Chemical structure of ingenol mebutate.



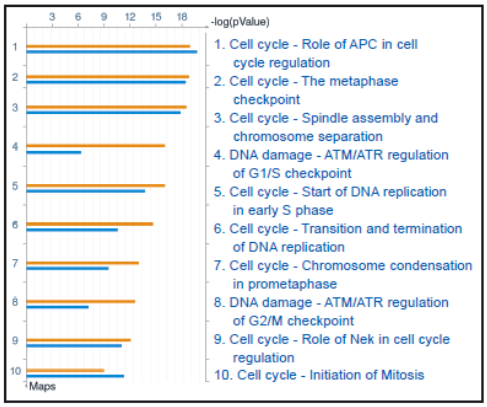
Supplementary Figure S2. Effect of IM on fibroblasts and melanoma cells and effect of TPA on SCC13 cells. A, Fibroblasts and melanoma cells were treated for 24h with indicated concentrations of IM followed by assessing cell viability by MTT assay. Graphs represent mean and standard deviation of three independent

experiments from two different fibroblast or melanoma cell cultures. B, SCC13 cells were treated for 24h with indicated concentrations of TPA followed by assessing cell viability by MTT assay. Graphs represent mean and SD of three independent experiments.

A



B

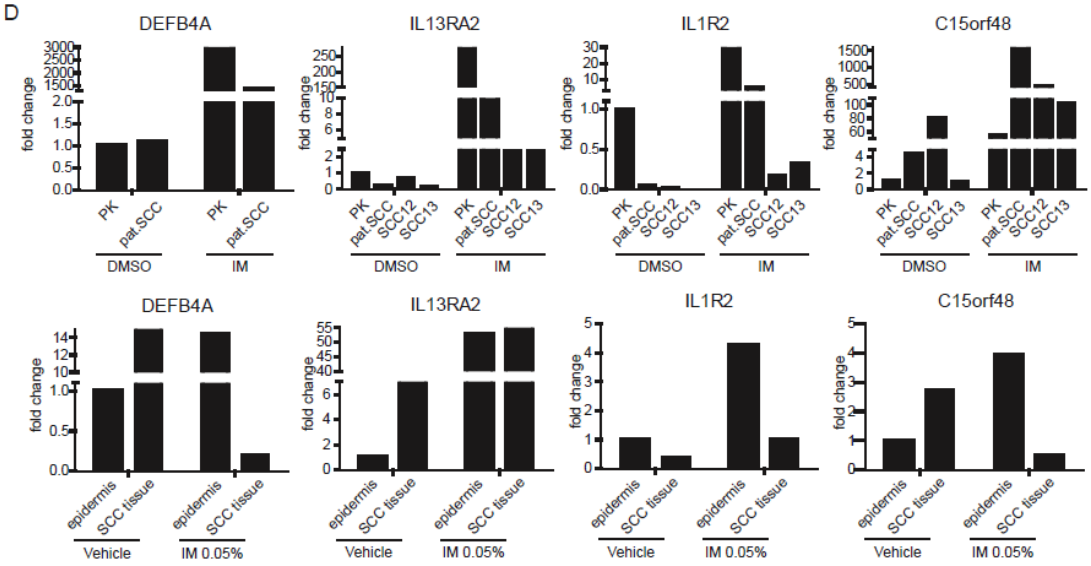


C

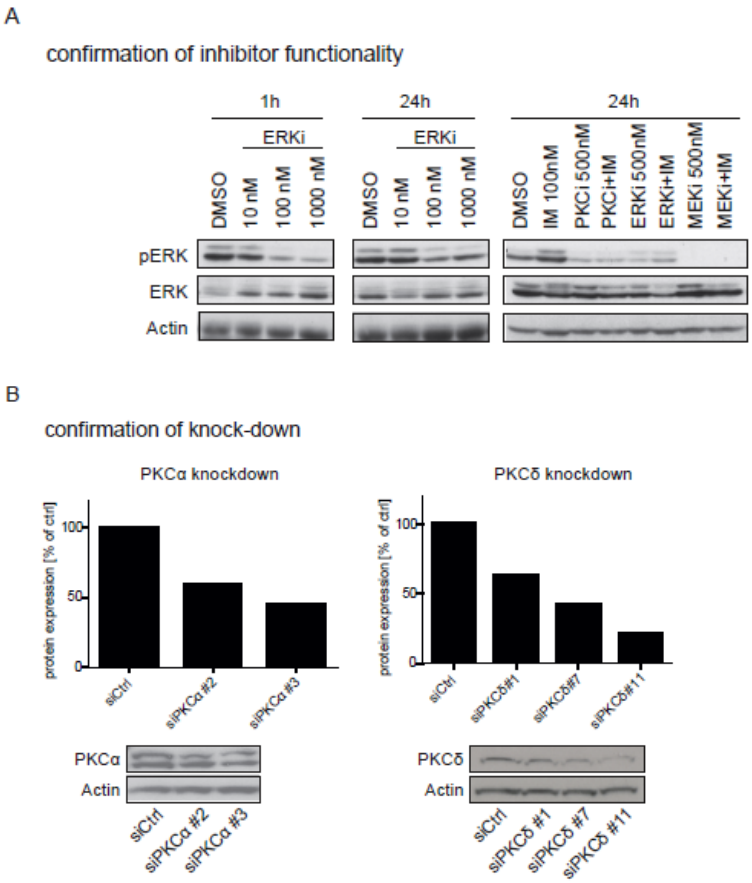
Network Objects	# of Maps containing Network Object	Network Objects ranking Overall	PK 100nM
ERK1/2	31	1	1
c-Fos	25	2	2
SOS	20	3	3
Calmodulin	15	4	4
IP3receptor	15	4	4
PKC-delta	11	5	5

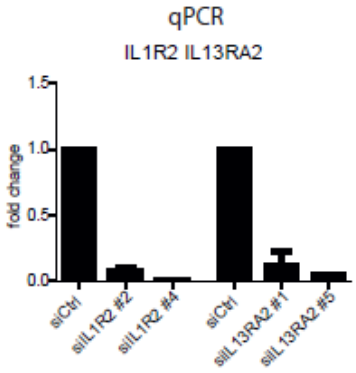
Network Objects	# of Maps containing Network Object	Network Objects ranking Overall	SCC 100nM
ERK1/2	30	1	1
I-kB	17	2	2
ICAM-1	10	3	3
MMP1	9	4	4
EGFR	8	5	5

D

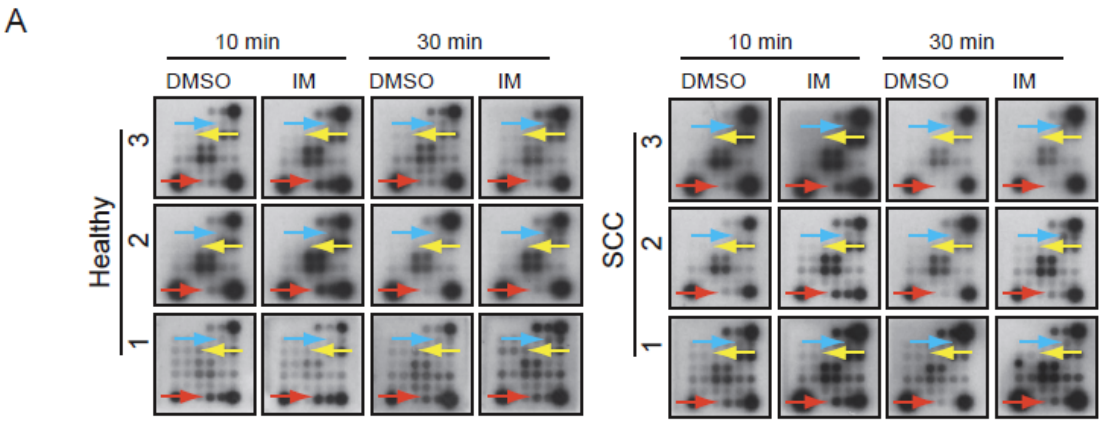


Supplementary Figure S3. Gene expression analysis of IM-treated primary keratinocytes and patient-derived SCC cells. A, Heatmap showing differentially expressed genes between DMSO-treated and IM-treated primary keratinocytes and patient-derived SCC cells. Cells were treated with either DMSO or 10 µM IM or DMSO and 1 nM IM respectively for 24h following RNA extraction and performance of the ¬microarray. Upregulated genes are displayed in red, downregulated genes are displayed in blue. B, GeneGo analysis of downregulated genes after treatment with 100 nM of IM. The top 10 pathways that are most affected by IM-dependent gene downregulation are cell cycle-associated. C, GeneGo analysis of upregulated genes after 100 nM of IM treatment. List of network objects that are most involved in processes affected by IM-dependent gene upregulation. D, Validation of selected genes from the microarray by qPCR. Primary keratinocytes, patient-derived SCC cells, SCC12 and SCC13 cells were treated for 24h with DMSO or 100 nM of IM and organ cultures from normal skin and SCC treated with either vehicle or IM 0.05% followed by RNA extraction. Gene expression was analyzed by qPCR.

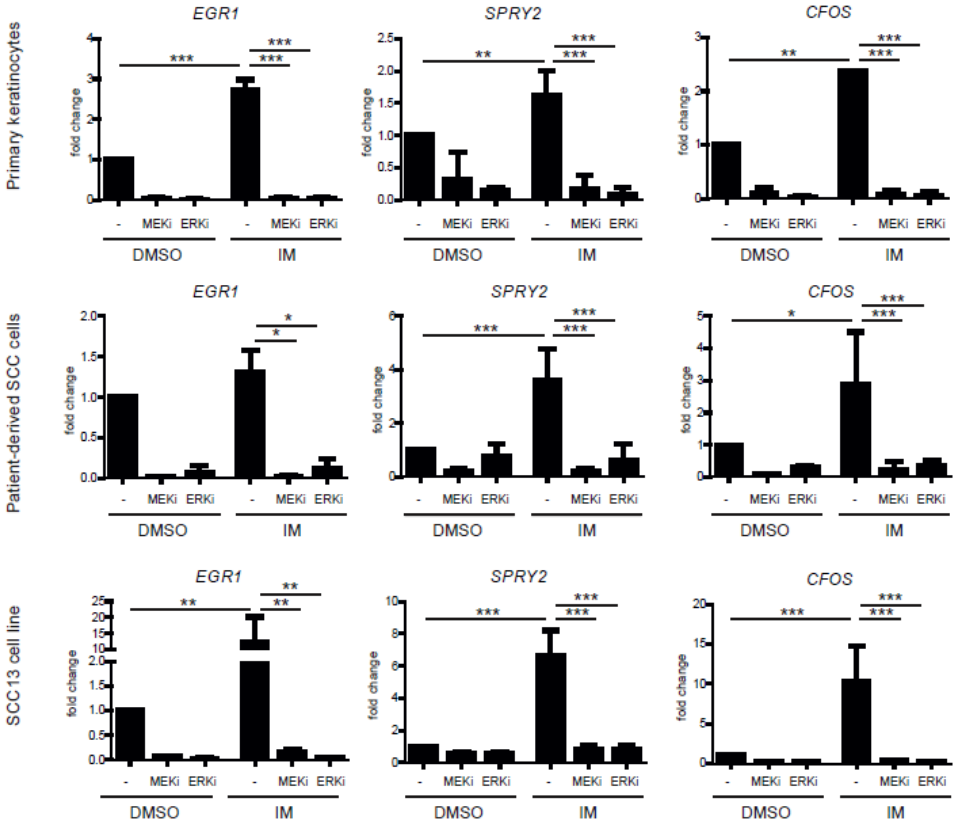




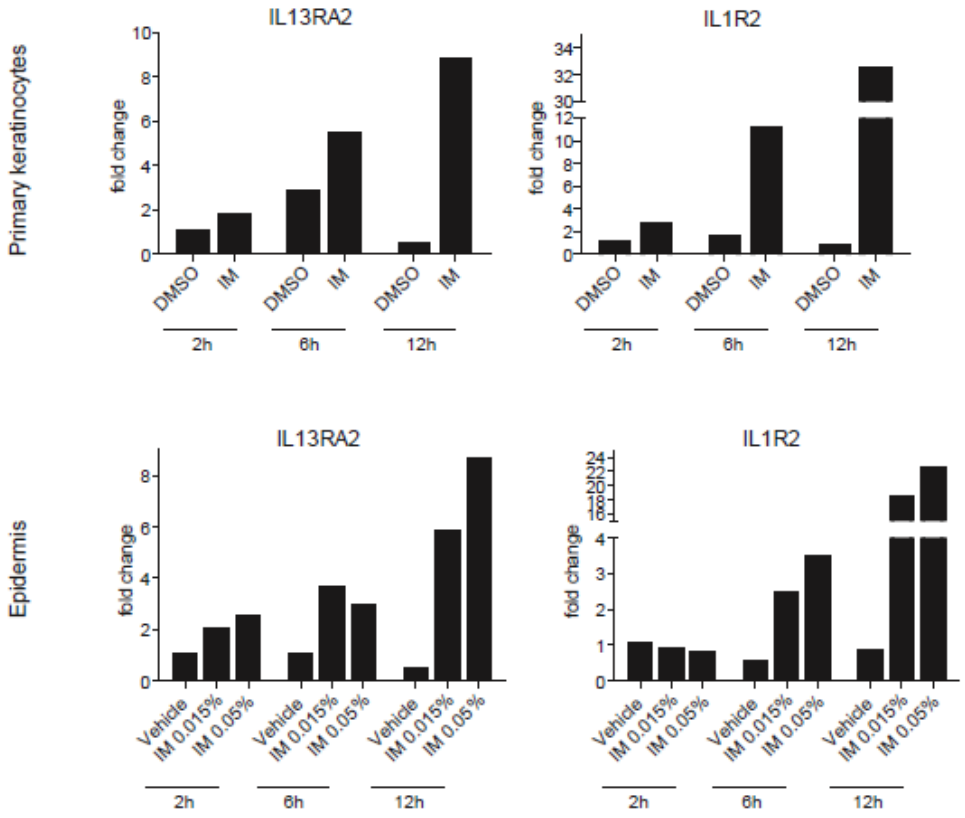
Supplementary Figure S4. Confirmation of inhibition by MEK and ERK inhibitor. A, Cells were treated with MEK and ERK inhibitor to confirm their inhibitory potential. WB for pERK confirmed the inhibitory effect. Conformation of knock-down for siRNA experiments. Cells were transfected with indicated siRNAs for 30h followed by protein extraction or RNA extraction. Knock-down was confirmed by western blotting or qPCR. Actin was used as loading control for western blotting. B, Confirmation of knock-down of PKCδ and PKCα and confirmation of knock-down of IL1R2 and IL13RA2.



B



Supplementary Figure S5. ERK is activated after IM treatment in a PKC-dependent manner. A, Primary keratinocytes and patient-derived SCC cells from three different donors were treated with either DMSO or 100 nM IM for 10 min or 30 min followed by protein extraction. Phosphorylation was detected with the PathScan Intracellular Signaling Kit. Antibody spots are present in duplicates. Red arrow = ERK, blue arrow = JNK, arrow mark = p38. B, ERK target gene expression was analyzed by qPCR after 24h of IM treatment +/- MEK or ERK inhibitor in primary keratinocytes and SCC cells and in SCC13 cells. All graphs represent mean +/- SD of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnet's multiple-comparison test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.



Supplementary Figure S6. Time course experiment for gene expression analysis of IL13RA2 and IL1R2. Primary keratinocytes and organ cultures of normal skin were treated with either 100 nM of IM or 0.015% or 0.05% of IM respectively for 2h, 6h or 12h followed by RNA extraction. Gene expression was analyzed by qPCR.

1.7. References

1. Ackerman AB, Mones JM. Solar (actinic) keratosis is squamous cell carcinoma. Br J Dermatol. 2006;155:9-22.

2. Glogau RG. The risk of progression to invasive disease. J Am Acad Dermatol. 2000;42:23-4.

3. Brash DE, Rudolph JA, Simon JA, Lin A, McKenna GJ, Baden HP, et al. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci U S A. 1991;88:10124-8.

4. Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. N Engl J Med. 2003;348:1681-91.

5. Hofbauer GF, Attard NR, Harwood CA, McGregor JM, Dziunycz P, Iotzova-Weiss G, et al. Reversal of UVA skin photosensitivity and DNA damage in kidney transplant recipients by replacing azathioprine. Am J Transplant. 2012;12:218-25.

6. Wu X, Nguyen BC, Dziunycz P, Chang S, Brooks Y, Lefort K, et al. Opposing roles for calcineurin and ATF3 in squamous skin cancer. Nature. 2010;465:368-72.

7. Dziunycz PJ, Lefort K, Wu X, Freiberger SN, Neu J, Djerbi N, et al. The oncogene ATF3 is potentiated by cyclosporine A and ultraviolet light A. J Invest Dermatol. 2014;134:1998-2004.

8. Hollestein LM, de Vries E, Nijsten T. Trends of cutaneous squamous cell carcinoma in the Netherlands: increased incidence rates, but stable relative survival and mortality 1989-2008. Eur J Cancer. 2012;48:2046-53.

9. Lazareth V. Management of non-melanoma skin cancer. Semin Oncol Nurs. 2013;29:182-94.

10. Lebwohl M, Swanson N, Anderson LL, Melgaard A, Xu Z, Berman B. Ingenol mebutate gel for actinic keratosis. N Engl J Med. 2012;366:1010-9.

11. Siller G, Gebauer K, Welburn P, Katsamas J, Ogbourne SM. PEP005 (ingenol mebutate) gel, a novel agent for the treatment of actinic keratosis: results of a randomized, double-blind, vehicle-controlled, multicentre, phase IIa study. Australas J Dermatol. 2009;50:16-22.

12. Siller G, Rosen R, Freeman M, Welburn P, Katsamas J, Ogbourne SM. PEP005 (ingenol mebutate) gel for the topical treatment of superficial basal cell carcinoma: results of a randomized phase IIa trial. Australas J Dermatol. 2010;51:99-105.

13. Ogbourne SM, Suhrbier A, Jones B, Cozzi SJ, Boyle GM, Morris M, et al. Antitumor activity of 3-ingenyl angelate: plasma membrane and mitochondrial disruption and necrotic cell death. Cancer Res. 2004;64:2833-9.

14. Challacombe JM, Suhrbier A, Parsons PG, Jones B, Hampson P, Kavanagh D, et al. Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with ingenol-3-angelate. J Immunol. 2006;177:8123-32.

15. Ghoul A, Serova M, Astorgues-Xerri L, Bieche I, Bousquet G, Varna M, et al. Epithelial-to-mesenchymal transition and resistance to ingenol 3-angelate, a novel protein kinase C modulator, in colon cancer cells. Cancer Res. 2009;69:4260-9.

16. Cozzi SJ, Parsons PG, Ogbourne SM, Pedley J, Boyle GM. Induction of senescence in diterpene ester-treated melanoma cells via protein kinase C-dependent hyperactivation of the mitogen-activated protein kinase pathway. Cancer Res. 2006;66:10083-91.

17. Olsnes AM, Ersvaer E, Ryningen A, Paulsen K, Hampson P, Lord JM, et al. The protein kinase C agonist PEP005 increases NF-kappaB expression, induces

differentiation and increases constitutive chemokine release by primary acute myeloid leukaemia cells. Br J Haematol. 2009;145:761-74.

18. Stahlhut M, Bertelsen M, Hoyer-Hansen M, Svendsen N, Eriksson AH, Lord JM, et al. Ingenol mebutate: induced cell death patterns in normal and cancer epithelial cells. J Drugs Dermatol. 2012;11:1181-92.

19. Rheinwald JG, Beckett MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. Cancer Res. 1981;41:1657-63.

20. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:Article3.

21. Long X, Tharp DL, Georger MA, Slivano OJ, Lee MY, Wamhoff BR, et al. The smooth muscle cell-restricted KCNMB1 ion channel subunit is a direct transcriptional target of serum response factor and myocardin. J Biol Chem. 2009;284:33671-82.

22. Kedei N, Lundberg DJ, Toth A, Welburn P, Garfield SH, Blumberg PM. Characterization of the interaction of ingenol 3-angelate with protein kinase C. Cancer Res. 2004;64:3243-55.

23. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. Nat Med. 2006;12:99-106.

24. Kawakami K, Kawakami M, Snoy PJ, Husain SR, Puri RK. In vivo overexpression of IL-13 receptor alpha2 chain inhibits tumorigenicity of human breast and pancreatic tumors in immunodeficient mice. J Exp Med. 2001;194:1743-54.

25. Lee CH, Chang JS, Syu SH, Wong TS, Chan JY, Tang YC, et al. IL-1beta promotes malignant transformation and tumor aggressiveness in oral cancer. J Cell Physiol. 2015;230:875-84.

26. Yoshimoto T, Yamaai T, Mizukawa N, Sawaki K, Nakano M, Yamachika E, et al. Different expression patterns of beta-defensins in human squamous cell carcinomas. Anticancer Res. 2003;23:4629-33.

27. Steinberg SF. Distinctive activation mechanisms and functions for protein kinase Cdelta. Biochem J. 2004;384:449-59.

28. Liu Y, Belkina NV, Graham C, Shaw S. Independence of protein kinase C-delta activity from activation loop phosphorylation: structural basis and altered functions in cells. J Biol Chem. 2006;281:12102-11.

29. Dieckgraefe BK, Weems DM. Epithelial injury induces egr-1 and fos expression by a pathway involving protein kinase C and ERK. Am J Physiol. 1999;276:G322-30.

30. Benhadji KA, Serova M, Ghoul A, Cvitkovic E, Le Tourneau C, Ogbourne SM, et al. Antiproliferative activity of PEP005, a novel ingenol angelate that modulates PKC functions, alone and in combination with cytotoxic agents in human colon cancer cells. Br J Cancer. 2008;99:1808-15.

31. Zhang H, Okamoto M, Panzhinskiy E, Zawada WM, Das M. PKCdelta/midkine pathway drives hypoxia-induced proliferation and differentiation of human lung epithelial cells. Am J Physiol Cell Physiol. 2014;306:C648-58.

32. Papp H, Czifra G, Bodo E, Lazar J, Kovacs I, Aleksza M, et al. Opposite roles of protein kinase C isoforms in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes. Cell Mol Life Sci. 2004;61:1095-105.

33. Hernandez-Maqueda JG, Luna-Ulloa LB, Santoyo-Ramos P, Castaneda-Patlan MC, Robles-Flores M. Protein kinase C delta negatively modulates canonical Wnt pathway and cell proliferation in colon tumor cell lines. PLoS One. 2013;8:e58540.

34. Bowles DK, Maddali KK, Dhulipala VC, Korzick DH. PKCdelta mediates anti-proliferative, pro-apoptic effects of testosterone on coronary smooth muscle. Am J Physiol Cell Physiol. 2007;293:C805-13.

35. Serova M, Ghoul A, Benhadji KA, Faivre S, Le Tourneau C, Cvitkovic E, et al. Effects of protein kinase C modulation by PEP005, a novel ingenol angelate, on mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling in cancer cells. Mol Cancer Ther. 2008;7:915-22.

36. Pritchard AL, Hayward NK. Molecular pathways: mitogen-activated protein kinase pathway mutations and drug resistance. Clin Cancer Res. 2013;19:2301-9.

37. Deschenes-Simard X, Gaumont-Leclerc MF, Bourdeau V, Lessard F, Moiseeva O, Forest V, et al. Tumor suppressor activity of the ERK/MAPK pathway by promoting selective protein degradation. Genes Dev. 2013;27:900-15.

38. Svensson S, Jirstrom K, Ryden L, Roos G, Emdin S, Ostrowski MC, et al. ERK phosphorylation is linked to VEGFR2 expression and Ets-2 phosphorylation in breast cancer and is associated with tamoxifen treatment resistance and small tumours with good prognosis. Oncogene. 2005;24:4370-9.

39. Bernard J, Treton D, Vermot-Desroches C, Boden C, Horellou P, Angevin E, et al. Expression of interleukin 13 receptor in glioma and renal cell carcinoma: IL13Ralpha2 as a decoy receptor for IL13. Lab Invest. 2001;81:1223-31.

40. Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, et al. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science. 1993;261:472-5.

41. Khoufache K, Bondza PK, Harir N, Daris M, Leboeuf M, Mailloux J, et al. Soluble human IL-1 receptor type 2 inhibits ectopic endometrial tissue implantation and growth: identification of a novel potential target for endometriosis treatment. Am J Pathol. 2012;181:1197-205.

2. S100A8/A9 stimulates keratinocyte proliferation in the development of squamous cell carcinoma of the skin via the receptor for advanced glycation-end products

The receptor for advanced glycation end products (RAGE) binds advanced glycosylation end products (AGEs) and was found to be expressed on keratinocytes. Besides AGEs, the S100 proteins serve as ligands for the receptor, linking it to inflammatory processes. However, a role for RAGE in tumor development was reported as well. In several studies decreased RAGE expression was associated with tumor growth inhibition. In this project we investigated the impact of S100A8/A9 and its binding to RAGE on the development of SCC.

Contribution to the manuscript:
I contributed to this project by isolating primary cells from tissue and by performing proliferation assays and protein analysis by western blotting. Furthermore I participated in experiment planning and discussion.

This paper is published in PLOS ONE.

PLOS | ONE

RESEARCH ARTICLE

S100A8/A9 Stimulates Keratinocyte Proliferation in the Development of Squamous Cell Carcinoma of the Skin via the Receptor for Advanced Glycation-End Products

Guergana Iotzova-Weiss[1]*, Piotr J. Dziunycz[1], Sandra N. Freiberger[1], Severin Läuchli[1], Jürg Hafner[1], Thomas Vogl[2], Lars E. French[1], Günther F. L. Hofbauer[1]

1 Department of Dermatology, University Hospital Zurich, Zurich, Switzerland, 2 Institute of Immunology, University Clinic Münster, Münster, Germany

* geri.weiss@web.de

Citation: Iotzova-Weiss G, Dziunycz PJ, Freiberger SN, Läuchli S, Hafner J, Vogl T, et al. (2015) S100A8/A9 Stimulates Keratinocyte Proliferation in the Development of Squamous Cell Carcinoma of the Skin via the Receptor for Advanced Glycation-End Products. PLoS ONE 10(3): e0120971. doi:10.1371/journal.pone.0120971

Academic Editor: Barry I Hudson, University of Miami, UNITED STATES

Received: September 19, 2014

Accepted: January 27, 2015

Published: March 26, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by SCC001 EMDO Stiftung (http://www.researchers.uzh.ch/static/fnfdstiftungen/stiftung.php?id=173), and SCC002 Olga Mayenfisch Stiftung (http://www.med.uzh.ch/StiftungenundPreise/olgamayenfischstiftung.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Squamous cell carcinoma (SCC) is the most common neoplasm in organ transplant recipients (OTR) on long-term immunosuppression and occurs 60- to 100-fold more frequently than in the general population. Here, we present the receptor for advanced glycation end products (RAGE) and S100A8/A9 as important factors driving normal and tumor keratinocyte proliferation. RAGE and S100A8/A9 were transcriptionally upregulated in SCC compared to normal epidermis, as well as in OTR compared to immunocompetent patients (IC) with SCC. The proliferation of normal and SCC keratinocytes was induced by exposure to exogenous S100A8/A9 which in turn was abolished by blocking of RAGE. The migratory activities of normal and SCC keratinocytes were also increased upon exposure to S100A8/A9. We demonstrated that exogenous S100A8/A9 induces phosphorylation of p38 and SAPK/JNK followed by activation of ERK1/2. We hypothesize that RAGE and S100A8/A9 contribute to the development of human SCC by modulating keratinocyte growth and migration. These processes do not seem to be impaired by profound drug-mediated immunosuppression in OTR.

Introduction

Squamous cell carcinoma is a common skin neoplasm characterized by infiltrative, destructive growth and metastasis. It is the most common malignant neoplasm in organ transplant recipients on long-term immunosuppression and occurs 60- to 100-fold more frequently than in the general population [1]. The early recognition of SCC is important because the neoplasm may acquire the ability to metastasize. Actinic keratoses (AKs) are considered by some as precancerous lesions, while others consider them an incipient form of SCC [2]. Studies have

Competing Interests: The authors have declared that no competing interests exist.

demonstrated that approximately 8% of all AKs will progress to invasive SCC in the general population [3], and potentially more in OTR. Recognition and treatment of AK are important for the prevention of neoplasm progression. It is well known that AK is surrounded by a peritumoral inflammatory infiltrate before development of invasive SCC, also observed in OTR under immunosuppression [4–5]. Anti-tumour defence by the immune system seems to play an important role on one side. On the other side, chronic sustained inflammation seems to create a pro-tumorigenic environment [6]. Such smoldering inflammatory mechanisms in the skin may be at least in part mediated by RAGE and S100A8/A9 [7–10].

RAGE is a multi-ligand member of the immunoglobulin superfamily of cell surface molecules [11–13] and is implicated in inflammation and cancer [14–18]. RAGE ligation activates important signal transduction pathways involved in tumorigenesis and inflammatory responses such as the mitogen activated protein kinase (MAPK) family (p38, Erk1/2 and JNK) and Rho GTPases (cdc42 and rac) [19–22]. To the spectrum of RAGE ligands belongs the S100 family of proteins (calgranulins) including S100A12 [23], S100A9 [24–26] and S100A8/A9 heterodimer [19, 27, 28]. They activate cellular processes and cell migration, and have properties similar to proinflammatory cytokines [29–32]. S100A8 and S100A9 are secreted by neutrophils and activated monocytes [33–34] and induce activation of NF-κB [31, 35, 36]. They are associated with chronic inflammation [37–39] and cancer [10, 39–43].

Up to now the role of RAGE-S100A8/A9 signaling in keratinocytes in SCC formation has not been investigated. Here, we analyze the involvement of these proteins in the development of human SCC.

Results

RAGE, S100A8 and S100A9 are expressed in SCC of OTRs and IC

Epidermal mRNA expression of S100A8 and S100A9 differs between OTR and IC with invasive SCC. The mRNA expression level of RAGE in whole skin was similar in normal skin, IC SCC and OTR SCC. S100A8 and S100A9 mRNA expression was increased only in SCC of IC versus normal skin (Fig. 1A). In the epidermal fraction, however, the expression of RAGE, S100A8 and S100A9 was increased in invasive as well as in in-situ SCC of IC and OTR, all compared to normal epidermis (Fig. 1B).

Expression of RAGE and S100A8/A9 in SCC and normal skin on protein level. The expression of RAGE and the S100A8/A9 complex on protein level was analyzed by immunohistochemistry. Although the analysis is semi-quantitative, both proteins were clearly upregulated in invasive or in situ SCC of IC and OTR in comparison to healthy skin (Fig. 2). We did not detect a differential protein expression of RAGE and S100A8/A9 between the OTR and IC groups.

Endogenous S100A8/A9 is involved in cellular proliferation

Using ELISA specific for S100A8/A9, we detected that normal keratinocytes secrete S100A8/A9. In comparison to SCC-derived keratinocytes, normal keratinocytes showed lower levels of spontaneous S100A8/A9 secretion (Fig. 3A). The direct blockade of RAGE using a specific neutralizing anti-RAGE antibody resulted in a reduction of cellular proliferation by 20–25% (Fig. 3B). This suggests that the endogenous production of S100A8/A9 and RAGE signaling contributes to keratinocyte proliferation.

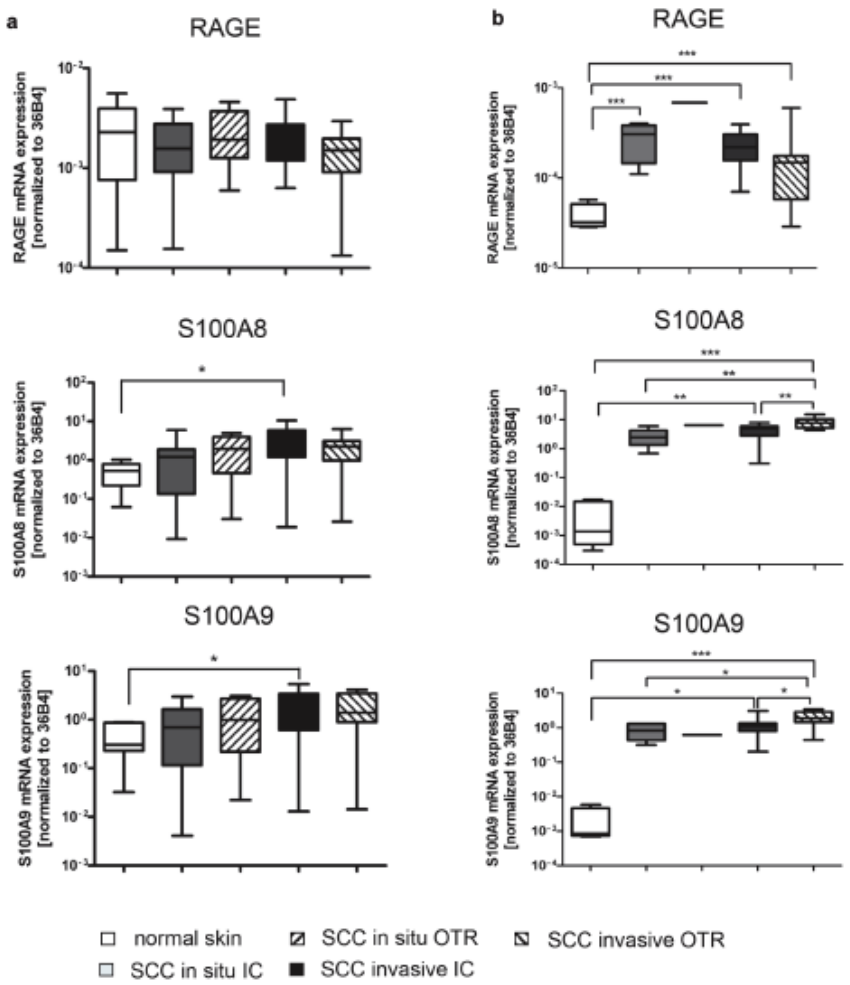


Fig 1. Differential expression of RAGE, S100A8 and S100A9 on transcriptional level in whole skin and epidermis. Expression of RAGE, S100A8 and S100A9 on transcriptional level. The expression was assessed by real-time PCR using specific primers for RAGE, S100A8 and S100A9. The expression level was normalized to 36B4 housekeeping gene. a: whole skin: No significant difference of RAGE expression was detected within or among the groups.

S100A8 and S100A9 mRNAs are increased only in IC patients with invasive SCC versus normal skin (*p<0.05). b: epidermis: The expression of RAGE, S100A8 and S100A9 transcripts was significantly increased in OTR and IC patients with invasive or in situ SCC versus normal epidermis (** *p<0.001). Significant difference in the expression of S100A8 and S100A9 was also detected between the IC and OTR groups with invasive SCC (*p<0.05; **p<0.01).

doi:10.1371/journal.pone.0120971.g001

Keratinocytes proliferate in response to exogenous S100A8/A9

Cells were seeded in 96 well plates and exposed for 24 hours to purified S100A8/A9 at concentrations between 0.01–1μg/ml. All normal, AK and SCC-derived primary keratinocytes responded with increased proliferation ranging from 30% to 70% after 24 hours exposure to S100A8/A9 as revealed by BrdU assay (Fig. 4A). Induced BrdU incorporation by S100A8/A9 was also detected by flow cytometry (Fig. 4B). The total RAGE expression was analyzed by FACS and western blotting (S1 Fig. A, B).

Direct blockade and knockdown of RAGE reduce cellular proliferation

Keratinocyte proliferation following S100A8/A9 exposure was abolished by blockade of RAGE using a functional blocking antibody (Fig. 5A). A reduction of cellular growth by 80% was detected also when shRNA specific for RAGE was used (Fig. 5B). Proliferation was not influenced by stimulation of shRAGE-transfected keratinocytes with recombinant S100A8/A9. Using the antibody HTA125 blocking TLR4, a potentially important receptor for S100A8/A9, proliferation was not impaired following exposure of keratinocytes to S100A8/A9 (Fig. 5C).

S100A8/A9 induces migration of normal and SCC-derived keratinocytes

The effect of S100A8/A9 on the ability of normal and SCC-derived keratinocytes to migrate *in vitro* was investigated using scratch assay. An increase in the number of migrated cells was detected 15 h after scratching (between 60–100%) in both normal and SCC-derived keratinocytes in the presence of 0.01 and 0.1μg/ml S100A8/A9 (Fig. 6 A, B). At 24 hours after scratching, the difference was no longer observed.

Exogenous S100A8/A9 induces RAGE surface expression and MAPK phosphorylation

Exogenous S100A8/A9 induced rapid and transient increase in RAGE surface expression 30 min after S100A8/A9 stimulation and reduced it 1 hour after induction (Fig. 7A), which underlines the stimulation of S100A8/A9 through RAGE. To clarify the mechanism of S100A8/A9 we further investigated the phosphorylation of p38, ERK1/2 and JNK/SAPK after stimulation with S100A8/A9 (Fig. 7B). SCC-derived keratinocytes showed a prolonged activation of p38 phosphorylation in comparison to normal keratinocytes whereas the phosphorylation of p38 was slightly decreased 15 min after treatment. The phosphorylation of ERK1 decreased slightly 15–30 min after exposure to S100A8/A9 in both PK and SCC in comparison to unstimulated cells (Fig. 7B, S2 Fig.). An increase in the phosphorylation rate of ERK 1 was detected at later time intervals (45–60 min). SCC cells reacted with a phosphorylation of SAPK/JNK 15 minutes after exposure to S100A8/A9, whereas in normal keratinocytes phosphorylation of SAPK/JNK was not detected.

Discussion

Normal whole skin and fully excised SCC express RAGE, S100A8 and S100A9 mRNA in similar amounts, with only minor increases for S100A8 and S100A9 in IC SCC, while other inflammatory factors such as NFkB, CCL3, CCL2, Cox2, TGFß and MMP9 do differ from normal

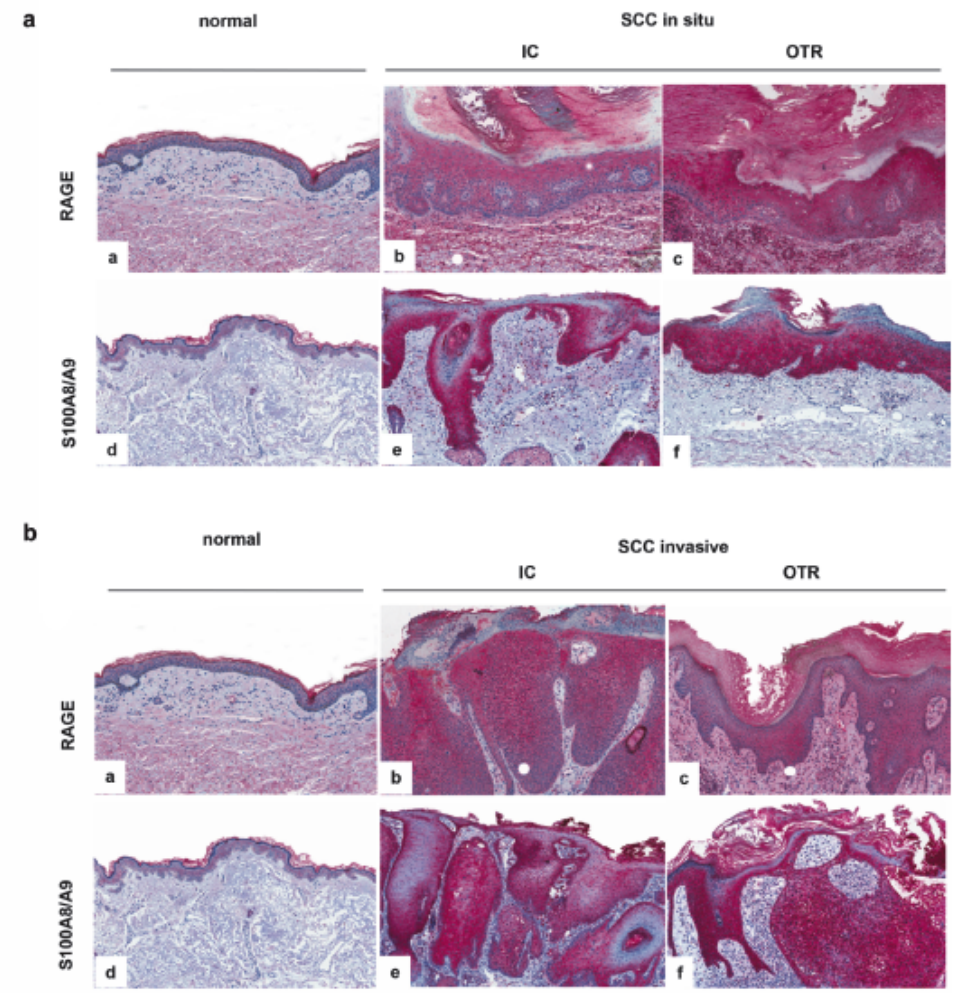


Fig 2. Differential expression of RAGE and S100A8/A9 on protein level in whole skin. The expression level was analysed by immunochistochemistry using specific anti human S100A8/A9 and anti human RAGE antibodies. The expression was tested in 5 patient samples per group (IC and OTR with in situ or invasive SCC). a: Expression of RAGE and S100A8/A9 in IC and OTR patients with in situ SCC in comparison to normal skin. b: Expression of RAGE and S100A8/A9 in IC and OTR patient with invasive SCC in comparison to normal skin.

doi:10.1371/journal.pone.0120971.g002

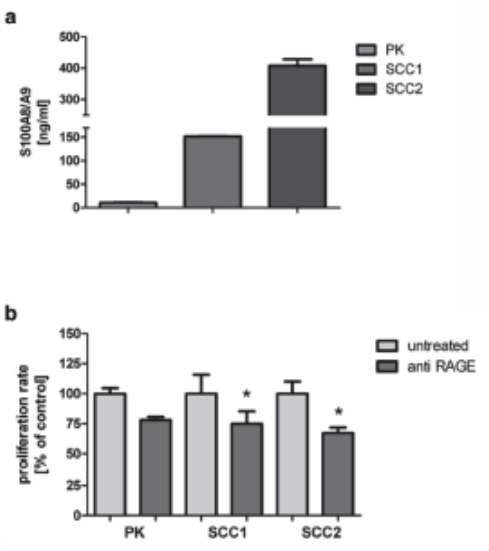


Fig 3. Endogenous S100A8/A9 is involved in cellular proliferation. a: Spontaneous secretion of S100A8/A9 from normal and SCC-derived keratinocytes. Normal and SCC-derived keratinocytes were grown in 96 well plates for 24 hours. Afterwards, supernatant was collected and preceded for the assessment of secreted S100A8/A9 using specific ELISA for S100A8/A9. b: Blockade of RAGE using anti RAGE blocking antibody reduces spontaneous proliferation of keratinocytes. Normal and SCC-derived keratinocytes were incubated with a blocking anti-RAGE antibody (8ug/100μl) for 24 hours. A decrease of the proliferation rate was detected (20–30%) based on the BrdU incorporation (t-test *p = 0.002). All the results are presented as percentage deviation from corresponding control and represent the mean +/- SD of duplicate values.

doi:10.1371/journal.pone.0120971.g003

skin to SCC (data not shown). This is probably due to the heterogeneous cell population present in whole skin. As reported, S100A8 and S100A9 are predominantly secreted by myeloid cells, but also by epithelial cells and keratinocytes during inflammation [27, 32, 33, 44–46]. In the epidermal compartment, however, both RAGE and S100A8 and S100A9 were strongly upregulated at mRNA and protein level already in intraepithelial lesions, suggesting an early role in SCC development. No additional difference was observed when comparing normal epidermis and SCC from IC and OTR patients, which suggests a role for RAGE and S100A8/A9 not impaired by commonly used immunosuppressive drugs. Previous studies in our group demonstrated that immunosuppressive agents such as Prednisolone and Cyclosporine are even able to induce S100A8/A9 expression in keratinocytes [43]. In support of our results, other reports showed that S100A8 and S100A9 were among the genes upregulated in SCC in comparison to normal skin [47]. Moreover, S100A8 and S100A9 were found to be upregulated in defined parts of dysplasia/cancer regions and in the invasion nests in SCC sections [48].

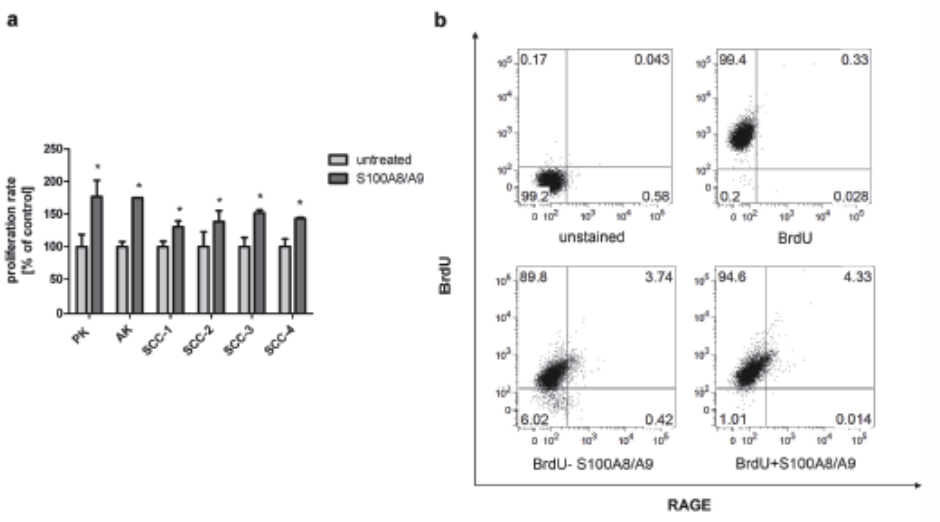


Fig 4. Exogenous S100A8/A9 induces keratinocyte proliferation. a: Assessment of the proliferation by BrdU assay. To assess the role of S100A8/A9 in normal primary, AK and SCC cultures, they were treated with purified S100A8/A9 for 24 hours and proliferation was assessed by BrdU incorporation. The induction of cellular proliferation was between 30–70% (2 way Anova, $p<0.0001$). b: Assessment of the proliferation by the incorporation of BrdU analysed by flow cytrometry. Normal keratinocytes were treated with S100A8/A9 (10ng/ml) and BrdU for 24 hours. Afterwards cells were fixed in 4% PFA, permeabilized with 0.5% Triton and stained for RAGE and BrdU using the antibodies mentioned above. The differential BrdU incorporation was compared to untreated cell.

doi:10.1371/journal.pone.0120971.g004

In summary, RAGE and its ligand S100A8/A9 seem active in SCC both in the setting of immunocompetence and immunosuppression, where immunosuppressive agents do not subdue the RAGE-S100A8/A9 axis but might potentially accentuate it.

To investigate the functional role of S100A8/A9 and RAGE, we generated primary keratinocyte cell cultures derived from normal skin and from lesions of patients with in situ (AK) or invasive SCC. Similar to other reports, we observed that low, rather than high concentrations of exogenous S100A8/A9 were sufficient to induce cellular proliferation [49, 50]. A potential function of S100A8/A9 as a driving factor for cell proliferation was reported in human breast cancer cells [19] and neonatal keratinocytes [51]. Correspondingly, we see S100A8/A9 as a driving factor for keratinocyte proliferation in human normal skin and SCC. Exogenous S100A8/A9 induced higher proliferation in normal keratinocytes in comparison to SCC keratinocytes unrelated to differential RAGE expression. We summarize that a differential dose-response relationship between the expression level of RAGE and S100A8/A9 and proliferation is hard to demonstrate due to several confounding factors such as origin of cells, number of cell passages, difference in cell signaling machinery between normal keratinocytes and different SCCs studied. Importantly, RAGE receptor blocking abolished the proliferative effect of exogenous S100A8/A9, indicating a critical role for RAGE. The possibility of additional receptors for S100A8/A9 on keratinocytes being involved such as TLR4 remains, as the secreted amount of

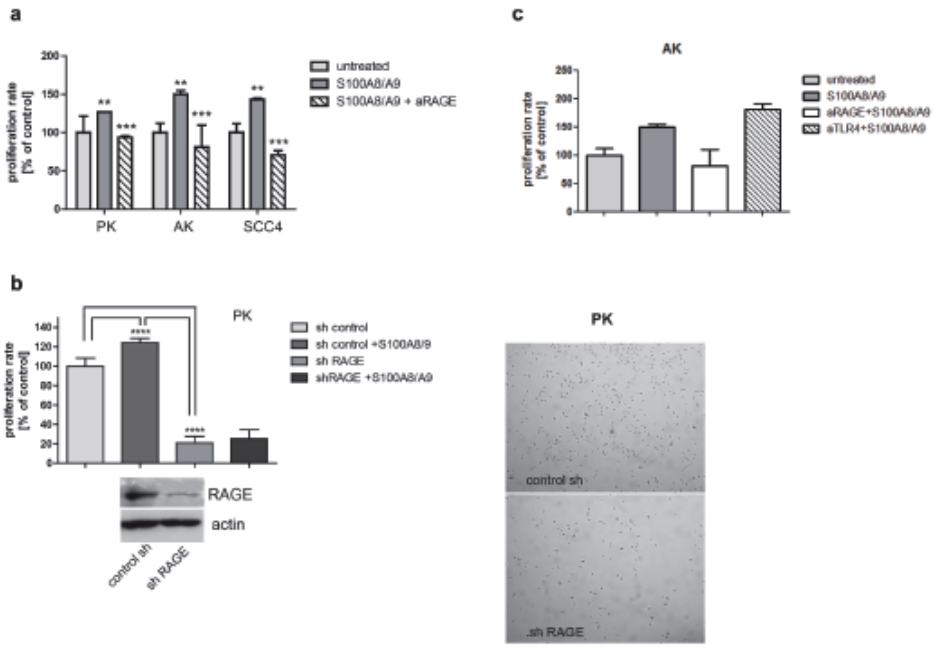


Fig 5. The receptor RAGE critically mediates the cellular response to S100A8/A9. a: Direct blockade by a specific RAGE blocking antibody reduces cellular proliferation. RAGE dependent proliferation was analyzed in normal primary, AK and SCC cells. Cells were incubated for 1 hour with a blocking anti-RAGE antibody (80μg/ml, as recommended by manufacturer) followed by S100A8/A9 stimulation for additional 24 hours. The differences in the proliferation after the blockade were assessed by BrdU incorporation (1 way Anova, Bonferroni's Multiple test, **p<0.05, ***p<0.05). b: Knockdown of RAGE using shRNA reduces cellular proliferation. The knockdown studies were performed using specific lentiviral shRNA against RAGE. Primary keratinocytes were infected by shRAGE and sh control viral particles. Selection of positive clones was performed by puromycine selection. Cells were grown to optimal confluence and were stimulated with 10ng/ml S100A8/A9. Cells with RAGE knockdown showed a delay in proliferation in comparison to control as assessed by BrdU (70%) (1 way Anova, Bonferroni's Multiple test p*** <0.0001) and microscope images. Exogenous S100A8/A9 did not induce proliferation of shRAGE keratinocytes, but only in the control. c: Blocking TLR4 using specific blocking antibody (HTA125) does not impair cellular proliferation. AK cells were treated with a specific blocking TLR4 antibody (HTA125). AK cells were grown for 24 hours in the presence of HTA125 antibody (1μg/ml) and S100A8/A9 (10ng/ml). For detection of the cellular proliferation rate BrdU proliferation assay was performed.

doi:10.1371/journal.pone.0120971.g005

S100A8/A9 from SCC cells was significantly higher than the decrease of the proliferation rate after RAGE blockade. However, we did not detect a decrease of cellular proliferation after the blockade of TLR4 by a specific blocking antibody. Our results correlate with other published data, where a relation between tumor growth and RAGE/S100A8/A9 axis was reported [15, 28]. Local production of S100A8/A9 within the epidermis may thus be a driving force for the proliferation of keratinocytes. Next to proliferation, our observation of increased migration suggests a role of S100A8/A9 in keratinocyte motility and a potential invasion.

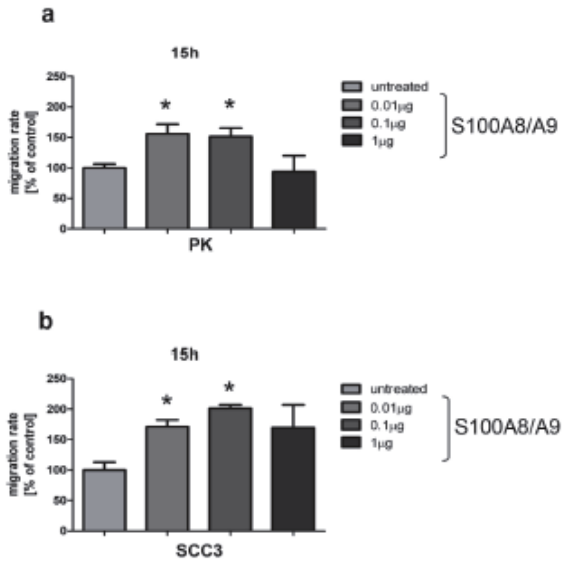


Fig 6. Exogenous S100A8/A9 induces migration of normal and SCC-derived primary keratinocytes. a, b: Cells were treated with different concentrations of purified S100A8/A9 (0.01–1 μg/ml) for 15h. The assessment of the migratory potential was analyzed by scratch assay where the number of migrated cells was analyzed. The migration of both normal and SCC-derived keratinocytes was increased significantly (between 60–100% depending on cell type) when 0.01 and 0.1 μg/ml of S100A8/A9 were used (t-test, PK, *p = 0.003, *p = 0.004; SCC, *p = 0.0014, *p = 0.0015).

doi:10.1371/journal.pone.0120971.g006

Normal and SCC-derived keratinocytes showed distinct phosphorylation of JNK after S100A8/A9 stimulation, which is probably due to a different response to S100A8/A9, based on their origin (tumor vs. normal cells). Studies concerning the role of RAGE-S100A8/A9 axis in the induction of MAPK, JNK or p38 pathways show that depending on the cell type, a differential phosphorylation pattern of these kinases is observed. In breast and colon cancer cells, S100A8/A9 is found to induce phosphorylation of ERK and JNK [19, 35, 52]. These results consider a role for ERK phosphorylation in RAGE-S100A8/A9 axis. Similarly, we observed phosphorylation of ERK in both normal and SCC keratinocytes and suggest that it may contribute to the proliferation and migration after S100A8/A9 stimulation. It is also possible that this kinase profile is due to a crosstalk between the MAPK, JNK and p38 pathways, regulating the balance between apoptosis (p38, JNK) and proliferation (ERK1/2) [53].

S100A8/A9 may act as a growth factor similar to chemokines. A chemokine-like function of S100A8/A9 was described in terms of enhancement of leukocyte recruitment to inflammatory sites [54–56]. Keratinocytes may be the initial source for S100A8/A9, in turn attracting inflammatory cells. The effect of S100A8/A9 on cell growth may be mediated by cytokine induction. Recently, it was reported that exogenous S100A8/A9 induces the expression of inflammatory

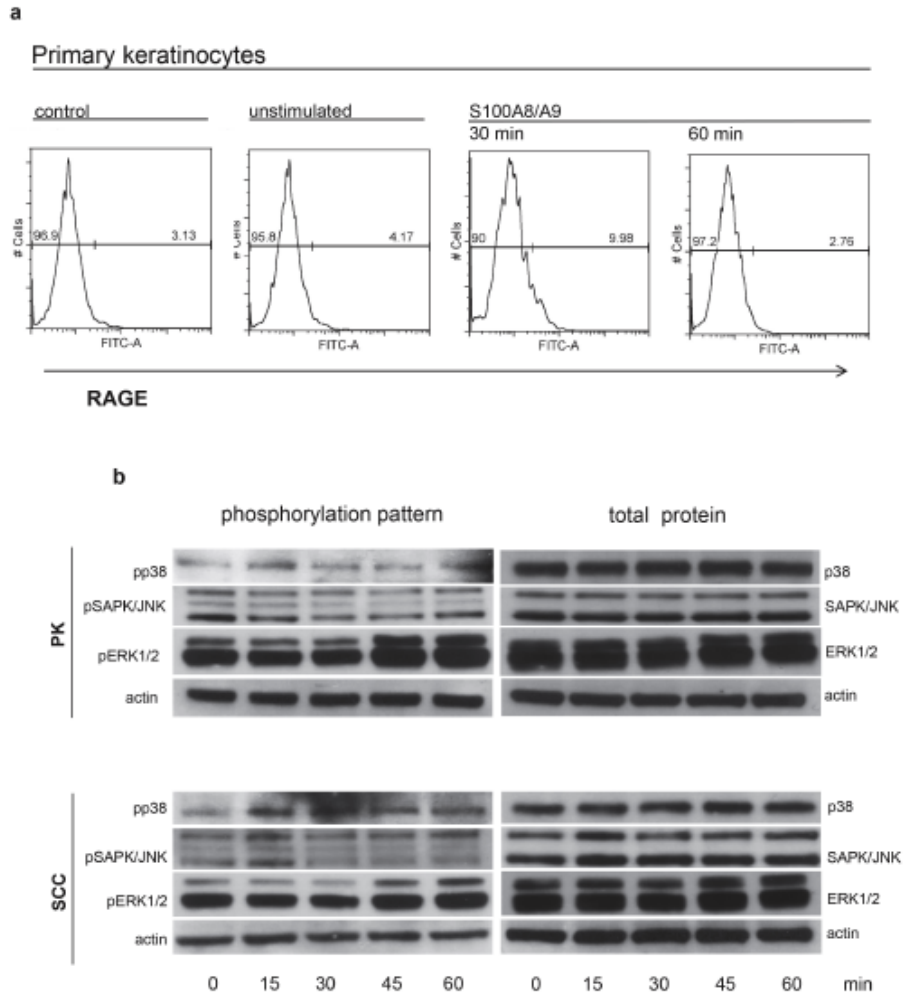


Fig 7. Exogenous S100A8/A9 induces RAGE surface expression and MAPK phosphorylation. a: Rapid and transient induction of RAGE surface expression after stimulation with S100A8/A9. Normal keratinocytes were induced with an exogenous S100A8/A9 (10ng/ml) in time intervals of 30 and 60 minutes. Afterwards cells were stained with the specific goat polyclonal anti RAGE antibody and a secondary RTC-conjugated antibody for analysis of both surface and total RAGE expression before and after S100A8/A9 treatment. Total expression of RAGE was analyzed prior to cell permeabilization with PFA and followed by blocking step with 1% BSA. As controls isotype control antibody and untreated cells were used. b: Exogenous S100A8/A9 induces

phosphorylation of MAPKs. Normal and SCC-derived keratinocytes were seeded in 6cm dishes in 50% confluence. Cells were starved for 18 h and stimulated afterwards with S100A8/A9 (10ng/ml) for different time intervals (0–60min). Cell lysates were collected after each time interval and the alteration of the phosphorylation of ERK1/2, p38 and SAPK/JNK was analyzed by western blot using the indicated antibodies.

doi:10.1371/journal.pone.0120971.g007

cytokines such as IL-8, IL-6 and TNF from neonatal keratinocytes [51] and macrophages [31, 34, 57]. While we hypothesize that S100A8/A9 influences the migration and invasion in SCC, its effect on metastasis remains controversial. Some reports show an inhibitory effect of S100A8/A9 on metastasis of human cervical cancer cells [58]. Clarifying the role of S100A8/A9 in the metastasis of SCC will be an important next step.

Organ transplant recipients suffer from SCC with a 60- to 100-fold increased incidence compared to the general population. In spite of their chronic immunosuppressive medication, a considerable amount of inflammation can still be observed in the local tumor microenvironment [4]. While low-level inflammation still occurs in the setting of chronic immunosuppression, tumor rejection by the immune system is impaired in organ transplant recipients, reflected by changes in the inflammatory microenvironment of SCC in OTR [59], [5]. We observed that the expression levels of RAGE and S100A8/A9 in OTR were still increased in comparison to healthy skin. It is tempting to speculate that S100A8/A9 induced by UV damage in sun-exposed skin could drive keratinocytes via RAGE to proliferate and migrate, eventually contributing to the greatly increased SCC formation in OTR.

In summary, we present evidence for a direct effect of S100A8/A9 on the proliferation and migration of normal human keratinocytes and keratinocytes originating directly from SCC lesions, suggesting a prominent role of S100A8/A9 on SCC formation, with RAGE as important target. This role of S100A8/A9 seems maintained in OTR. Targeting S100A8/A9 or RAGE may be of clinical benefit for SCC prevention and treatment.

Materials and Methods

Ethical considerations

The use of clinically indicated biopsy material for the study was approved by the ethical committee of the Canton of Zürich, Switzerland and was previously published: [60–64]

Tissue samples

Invasive SCC from organ transplant recipients (n = 13) and immunocompetent patients (n = 19) as well as in-situ SCC from OTRs and immunocompetent patients (1 and 5 respectively) were obtained at the time of surgery. Normal skin was obtained from abdominoplastic reconstructive surgery (n = 5). All specimens' diagnoses were confirmed by a board-certified dermatohistopathologist. 4mm punch biopsies from the SCC or normal skin area were placed in preheated PBS at 60°C for 45 seconds, and then chilled on ice in for 1 minute, followed by mechanical separation of epidermis and dermis. The epidermis was then homogenized in TRIzol Reagent (*Invitrogen, Basel, Switzerland*), and stored at −80°C

RNA extraction and reverse transcription. Total RNA was isolated using TRIzol Reagent following the instructions provided by the manufacturer. cDNA was synthesized using a Reverse Transcription System kit (*Promega, Dübendorf, Switzerland*) following the protocol provided. After 1:4 dilution with sterile water, cDNA was stored at −20°C and used as template for subsequent quantitative real-time polymerase chain reaction (RT-PCR).

Immunohistochemical analysis (IHC)

RAGE and S100A8/A9 protein expression was analyzed in formalin-fixed paraffin-embedded skin samples obtained from the archives of the Dermatology Department of University Hospital Zurich. The expression was tested in in-situ and invasive forms of SCC derived from OTRs and immunocompetent patients (5 samples per group)

Four μm thick sections were deparaffinized by 2x10 min incubation in Xylol and then rehydrated in a descending ethanol series (from 100% ethanol to PBS). For antigen retrieval, citrate buffer pH 6 (0.05% Tween 20) was pre-heated to 95°C, and the sections were kept at this temperature for 15 min. They were then allowed to slowly cool down to room temperature, and were then transferred to PBS. Blocking of nonspecific antibody binding was achieved by incubating the sections in PBS containing 2.5% of normal rabbit serum for RAGE staining or 2.5% normal goat serum for S100A8/A9. The staining was performed using anti-human RAGE polyclonal goat antibody (*R&D Systems Europe LtD*) and anti-human S100A8/A9 mouse monoclonal antibody (clone #27E10) (*BMA Biomedicals, Augst, Switzerland*) in a working dilution 1:50 (anti-RAGE) and 1:100 (anti-S100A8/9) in Antibody Diluent (*Dako, Hamburg, Germany*). Overnight incubation was performed in a humid chamber at 4°C. For detection of antibody binding to S100A8/A9 the Dako REAL Detection System AP/RED was used, and for detection of antibody biding to RAGE the Vectastain ABC-APKit (*Vector Laboratories, Burlingame, CA, USA*) was used according the manufacturers` protocol. The slides were briefly soaked in hematoxylin and then left under running tap water for 10 min. The slides were mounted with Mounting Medium (*Dako*).

Generation of primary keratinocyte cultures derived from healthy individuals and SCC patients

Single keratinocytes were isolated from 3–4mm punch biopsies following the standard protocol for generation of primary keratinocyte cultures (*CELLnTEC, Bern, Switzerland*). For sufficient separation of the epidermis and dermis the punch biopsies were incubate overnight at 4°C in keratinocyte medium (Progenitor Cell Targeted (PCT) epidermal keratinocyte medium CnT07 (*CELLnTec*) with Dispase II (*Roche*). Afterwards the epidermal part of the skin was incubated in trypsin (room temperature, 30 min) to insure efficient isolation of single keratinocytes. Freshly isolated cells were washed with medium and then transferred into CnT07 medium for a continuous incubation (37°C, 5%CO2).

RNA extraction and Real Time PCR

RNA was extracted using the TRIzol reagent (Invitrogen, Basel, Switzerland) following the protocol provided by the manufacturer. cDNA was synthesized from 0.5μg total RNA using a Reverse Transcription system kit (*Promega*). Specific primers for RAGE (*Quantitect Primer assay, Quiagen AG, Hombrechtikon, Switzerland*), S100A8 (fwd GGGAATTTCCATGCCGTCT, rev CCTTTTTCCTGATATACTGAGGAC), S100A9 (fwd CTGTGTGGCTCCTCGGCT, rev GCGTTCCAGCTGCGACAT), 36B4 (fwd GCAATGTTGCCAGTGTCTGT, rev GCCTTGACCTTTTCAGCAAG) and K14 (*Quantitect Primer assay, Quiagen*) were used. Real Time PCR was performed using Light Cycler FastStart DNA Master Sybr Green 1 kit (*Roche, Switzerland*).

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2000 and GraphPad Prism 5.0 for Windows. The gene expression by real-time RT-PCR was quantified using the comparative

threshold cycle (Ct) method [65]. Statistical evaluation was performed using the 2 way and one-way ANOVA test followed by Bonferroni's Multiple Comparison Test, or t-test. P values less than 0.05 were considered statistically significant.

Flow cytometry

FACS analysis. The assessment of the surface RAGE expression after induction with S100A8/A9 was performed using FACS analysis. Cells were detached using 4mM ETDA in PBS, washed with PBS, incubated with a specific goat anti-human RAGE antibody (*R&D Systems*) and visualized by specific donkey-anti goat FITC labeled secondary antibody (*Santa Cruz, Biotechnology*) in PBS at 4°C for 1h under gentle shaking conditions. The analysis was performed on 10 000 gated live cells together with a control (unstained and the secondary-FITC conjugated antibody) using FACSDIVA software (*BD Biosciences, Basel, Switzerland*).

Double staining for RAGE and BrdU was performed using the specific RAGE antibody and specific mouse anti BrdU antibody (*Millipore*). Before the staining cells were fixed with 4% PFA for 20min, followed by permeabilization with 0.5% Triton X, washed 3 times and proceeded for staining.

BrdU proliferation assay

Cells were seeded in 96 well plates in serum-free CnT07 medium in a cell density of 4.10^3/well. Incubation with purified S100A8/A9 protein was performed using concentrations between 0.01–1ug/ml for 24h. Blocking goat anti-human RAGE antibody (*R&D systems*) was added 1h prior to S100A8/A9 exposure. Cell proliferation was measured using the BrdU proliferation assay (*Millipore*) according to the manufacturer`s instruction. The percentage of cell proliferation was calculated using the equation: (mean OD of treated cells/mean OD of control cells) X 100. Purified S100A8/A9 protein was provided by Thomas Vogl, (Institute for Immunology, University of Münster, Germany), [31, 66]. Human S100A8/A9 was extracted from human granulocytes (c = 1,39 mg/ml, buffer: HBS, pH 7,4; source: granulocytes from 12 human buffy coats). No endotoxin contamination was detected by Limulus assay (LAL). The purity of the protein was confirmed by Coomassie blue staining after SDS electrophoresis.

Immunoblotting

Normal and SCC-derived keratinocytes were grown in 6cm dishes in 60% confluence. Afterwards cells were stimulated with S100A8/A9 (10ng/ml) for different time intervals (0–60 min). Cells were lysed in RIPA buffer (*conventional recipe*). Cell lysates were collected after each time interval and subjected to PAGE, followed by immunoblotting. The phosphorylation of ERK1/2, p38 and SAPK/JNK was detected by specific rabbit anti phospho-ERK1/2, -p38 and -SAPK JNK antibodies (*Cell Signaling Technology*). The non-phosphorylated form of the kinases were detected using specific rabbit anti- ERK1/2, p38 (*Cell SignallingTechnology*) *and anti SAPK/JNK (Santa Cruz, Biotechnology)*. The loading control actin was detected by specific anti actin antibody (*Santa Cruz, Biotechnology*).

RAGE expression in normal and SCC keratinocytes was confirmed by using of specific human anti RAGE antibody (*Millipore*).

RNA knockdown study

Primary keratinocytes were infected with RAGE sh lentiviral particals (sc-36374-v) and control sh viral particals (sc-108080), following the protocol conditions (*Santa Cruz Biotechnology*). Positive clones were selected by puromycine selection. Stimulation of shRAGE and sh control

expressing cells with S100A8/A9 protein was performed when the cells have reached an appropriate density. For determination of the effect of RAGE knockdown on cellular proliferation with a following S100A8/A9 stimulation, BrdU proliferation assay was performed.

Scratch assay

Normal and SCC-derived keratinocytes were grown in 24 well plates until they reached full confluence and starved for further 15 hours in basal keratinocyte medium without supplements (*CELLnTEC*). Scratches were performed using a blue pipette tip. Cells were washed afterwards 2 times with basal keratinocyte medium. Exposure to exogenous S100A8/A9 was performed in a dose dependent manner using concentrations between 0.01–1 μg/ml. Exposure was performed in triplicates for each concentration and control cells. After 15 and 24 hours, respectively, cells were fixed with 4% Paraformaldehyde (*Merck, Dietikon, Switzerland*), stained with Diff-Quik kit (*Medion-Diagnostics AG, Düdingen, Switzerland*), and counted in the whole scratch. The percentage of migrated cells was calculated as % of control.

Supporting Information

S1 Fig. RAGE expression in normal and SCC-derived keratinocytes. a: Surface and total expression of RAGE in normal keratinocytes. Surface RAGE expression was analyzed by FACS using specific anti RAGE antibody and FITC-conjugated secondary antibody. For intracellular staining cells were permeabilized (4%PFA) and blocked (1% BSA) prior staining with the specific RAGE antibody. As a control isotype control antibody was used. b: RAGE expression in normal and SCC derived keratinocytes. Cell lysates from primary normal and SCC keratinocytes were set to SDS PAGE electrophoresis and western blotting was performed using specific primary anti RAGE and secondary HRP-conjugated antibodies. As a loading control additional staining against actin was performed using specific anti actin antibody.
(TIF)

S2 Fig. Densitometrical quantification of phosphorylated ERK, JNK and p38 proteins versus control. a: Spot density-based quantification of pERK1/2 versus ERK1/2 and versus loading control (actin). b: Spot density-based quantification of pSAPK/JNK (pp54/p46) versus SAPK/JNK and versus loading control (actin). c: Spot density-based quantification of pp38 versus p38 and versus loading control (actin).
(TIF)

S3 Fig. RAGE expression in normal keratinocytes after RAGE knockdown by lentiviral shRNA. The RAGE expression after knockdown was analyzed on transcriptional level by qPCR using specific primers for RAGE and compared to sh control.
(TIF)

S4 Fig. S100A8/A9 protein purification. S100A8/A9 has been extracted from granulocytes of buffy coats. The purity, quantity and quantity of the extracted S100A8/A9 were analyzed by Coomasie blue staining after SDS gel electrophoresis.
(TIF)

Author Contributions

Conceived and designed the experiments: GIW GFH. Performed the experiments: GIW PJD SNF. Analyzed the data: GIW PJD SNF GFH. Contributed reagents/materials/analysis tools: TV SL JH. Wrote the paper: GIW PJD SNF TV GFH LEF.

References

1. Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. N Engl J Med. 2003; 348 (17):1681–91. PMID: 12711744
2. Ackerman AB, Mones JM. Solar (actinic) keratosis is squamous cell carcinoma. Br J Dermatol. 2006; 155(1):9–22. PMID: 16792746
3. Fuchs A, Marmur E. The kinetics of skin cancer: progression of actinic keratosis to squamous cell carcinoma. Dermatol Surg. 2007; 33(9):1099–101. PMID: 17760601
4. Muhleisen B, Petrov I, Gachter T, Kurrer M, Scharer L, Dummer R, et al. Progression of cutaneous squamous cell carcinoma in immunosuppressed patients is associated with reduced CD123+ and FOXP3+ cells in the peritneoplastic inflammatory infiltrate. Histopathology. 2009; 55(1):67–76. doi: 10.1111/j.1365-2559.2009.03324.x PMID: 19614769
5. Kosmidis M, Dziunycz P, Suarez-Farinas M, Muhleisen B, Scharer L, Lauchli S, et al. Immunosuppression affects CD4+ mRNA expression and induces Th2 dominance in the microenvironment of cutaneous squamous cell carcinoma in organ transplant recipients. J Immunother. 2010; 33(5):538–46. doi: 10.1097/CJI.0b013e3181cc2615 PMID: 20463564
6. Hofbauer GF, Bavinck JN, Euvrard S. Organ transplantation and skin cancer: basic problems and new perspectives. Exp Dermatol. 2010.
7. Tan TT, Coussens LM. Humoral immunity, inflammation and cancer. Curr Opin Immunol. 2007; 19 (2):209–16. PMID: 17276050
8. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer. 2006; 6(1):24–37. PMID: 16397525
9. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002; 420(6917):860–7. PMID: 12490959
10. Mueller MM. Inflammation in epithelial skin tumours: old stories and new ideas. Eur J Cancer. 2006; 42 (6):735–44. PMID: 16527478
11. Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC, et al. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. J Biol Chem. 1992; 267(21):14998–5004. PMID: 1378843
12. Miyata T, Hori O, Zhang J, Yan SD, Ferran L, Iida Y, et al. The receptor for advanced glycation end products (RAGE) is a central mediator of the interaction of AGE-beta2microglobulin with human mononuclear phagocytes via an oxidant-sensitive pathway. Implications for the pathogenesis of dialysis-related amyloidosis. J Clin Invest. 1996; 98(5):1088–94. PMID: 8787669
13. Shinohara M, Thornalley PJ, Giardino I, Beisswenger P, Thorpe SR, Onorato J, et al. Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. J Clin Invest. 1998; 101(5):1142–7. PMID: 9486985
14. Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, et al. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med. 2005; 83(11):876–86. PMID: 16133426
15. Gebhardt C, Riehl A, Durchdewald M, Nemeth J, Furstenberger G, Muller-Decker K, et al. RAGE signaling sustains inflammation and promotes tumor development. J Exp Med. 2008; 205(2):275–85. doi: 10.1084/jem.20070679 PMID: 18208974
16. Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest. 2001; 108(7):949–55. PMID: 11581294
17. Xu XC, Abuduhadeer X, Zhang WB, Li T, Gao H, Wang YH. Knockdown of RAGE inhibits growth and invasion of gastric cancer cells. European journal of histochemistry: EJH. 2013; 57(4):e36. doi: 10.4081/ejh.2013.e36 PMID: 24441189
18. Leibold JS, Riehl A, Hettinger J, Durben M, Hess J, Angel P. Keratinocyte-specific deletion of the receptor RAGE modulates the kinetics of skin inflammation in vivo. The Journal of investigative dermatology. 2013; 133(10):2400–6. doi: 10.1038/jid.2013.185 PMID: 23594567
19. Ghavami S, Rashedi I, Dattilo BM, Eshraghi M, Chazin WJ, Hashemi M, et al. S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. J Leukoc Biol. 2008; 83(6):1484–92. doi: 10.1189/jlb.0607397 PMID: 18339893
20. Huttunen HJ, Fages C, Rauvala H. Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the cytoplasmic domain of the receptor but different downstream signaling pathways. J Biol Chem. 1999; 274(28):19919–24. PMID: 10391939
21. Stern D, Yan SD, Yan SF, Schmidt AM. Receptor for advanced glycation endproducts: a multiligand receptor magnifying cell stress in diverse pathologic settings. Adv Drug Deliv Rev. 2002; 54(12):1615–25. PMID: 12453678

22. Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, et al. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. Nature. 2000; 405(6784):354–60. PMID: 10830965
23. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell. 1999; 97(7):889–901. PMID: 10399917
24. Bjork P, Bjork A, Vogl T, Stenstrom M, Liberg D, Olsson A, et al. Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. PLoS Biol. 2009; 7(4):e97. doi: 10.1371/journal.pbio.1000097 PMID: 19402754
25. McNeill E, Hogg N. S100A9 has a protective role in inflammation-induced skin carcinogenesis. International journal of cancer Journal international du cancer. 2014.
26. Bando M, Zou X, Hiroshima Y, Kataoka M, Ross KF, Shinohara Y, et al. Mechanism of interleukin-1alpha transcriptional regulation of S100A9 in a human epidermal keratinocyte cell line. Biochimica et biophysica acta. 2013; 1829(9):954–62. doi: 10.1016/j.bbagrm.2013.03.010 PMID: 23563247
27. Schonthaler HB, Guinea-Viniegra J, Wculek SK, Ruppen I, Ximenez-Embun P, Guio-Carrion A, et al. S100A8-S100A9 protein complex mediates psoriasis by regulating the expression of complement factor C3. Immunity. 2013; 39(6):1171–81. doi: 10.1016/j.immuni.2013.11.011 PMID: 24332034
28. Choi DK, Li ZJ, Chang IK, Yeo MK, Kim JM, Sohn KC, et al. Clinicopathological roles of S100A8 and S100A9 in cutaneous squamous cell carcinoma in vivo and in vitro. Archives of dermatological research. 2014.
29. Donato R. Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. Biochimica et biophysica acta. 1999; 1450(3):191–231. PMID: 10395934
30. Kerkhoff C, Klempt M, Sorg C. Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9). Biochimica et biophysica acta. 1998; 1448(2):200–11. PMID: 9920411
31. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nature medicine. 2007; 13(9):1042–9. PMID: 17767165
32. Kerkhoff C, Voss A, Scholzen TE, Averill MM, Zanker KS, Bornfeldt KE. Novel insights into the role of S100A8/A9 in skin biology. Exp Dermatol. 2012; 21(11):822–6. doi: 10.1111/j.1600-0625.2012.01571.x PMID: 22882537
33. Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. J Biol Chem. 1997; 272(14):9496–502. PMID: 9083090
34. Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. J Leukoc Biol. 2007; 81(1):28–37. PMID: 16943388
35. Hermani A, De Servi B, Medunjanin S, Tessier PA, Mayer D. S100A8 and S100A9 activate MAP kinase and NF-kappaB signaling pathways and trigger translocation of RAGE in human prostate cancer cells. Exp Cell Res. 2006; 312(2):184–97. PMID: 16297907
36. Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Yamamoto H, et al. The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis. Arthritis Res Ther. 2006; 8(3):R69. PMID: 16613612
37. Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int J Biochem Cell Biol. 2001; 33(7):637–68. PMID: 11390274
38. Foell D, Seeliger S, Vogl T, Koch HG, Maschek H, Harms E, et al. Expression of S100A12 (EN-RAGE) in cystic fibrosis. Thorax. 2003; 58(7):613–7. PMID: 12832680
39. Gebhardt C, Nemeth J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. Biochem Pharmacol. 2006; 72(11):1622–31. PMID: 16846592
40. Arai K, Takano S, Teratani T, Ito Y, Yamada T, Nozawa R. S100A8 and S100A9 overexpression is associated with poor pathological parameters in invasive ductal carcinoma of the breast. Curr Cancer Drug Targets. 2008; 8(4):243–52. PMID: 18537548
41. Turovskaya O, Foell D, Sinha P, Vogl T, Newlin R, Nayak J, et al. RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. Carcinogenesis. 2008; 29(10):2035–43. doi: 10.1093/carcin/bgn188 PMID: 18689872
42. Shen J, Abel EL, Riggs PK, Repass J, Hensley SC, Schroeder LJ, et al. Proteomic and pathway analyses reveal a network of inflammatory genes associated with differences in skin tumor promotion susceptibility in DBA/2 and C57BL/6 mice. Carcinogenesis. 2012; 33(11):2208–19. doi: 10.1093/carcin/bgs213 PMID: 22782996

43. Djerbi N, Dziunycz PJ, Reinhardt D, Iotzova-Weiss G, Hafner J, Lauchli S, et al. Influence of cyclosporin and prednisolone on RAGE, S100A8/A9, and NFkappaBexpression in human keratinocytes. JAMA dermatology. 2013; 149(2):236–7. doi: 10.1001/jamadermatol.2013.836 PMID: 23426492
44. Lagasse E, Clerc RG. Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. Mol Cell Biol. 1988; 8(6):2402–10. PMID: 3405210
45. Murao S, Collart F, Huberman E. A protein complex expressed during terminal differentiation of monomyelocytic cells is an inhibitor of cell growth. Cell Growth Differ. 1990; 1(10):447–54. PMID: 2278876
46. Rugtveit J, Haraldsen G, Hogasen AK, Bakka A, Brandtzaeg P, Scott H. Respiratory burst of intestinal macrophages in inflammatory bowel disease is mainly caused by CD14+L1+ monocyte derived cells. Gut. 1995; 37(3):367–73. PMID: 7590432
47. Haider AS, Peters SB, Kaporis H, Cardinale I, Fei J, Ott J, et al. Genomic analysis defines a cancer-specific gene expression signature for human squamous cell carcinoma and distinguishes malignant hyperproliferation from benign hyperplasia. The Journal of investigative dermatology. 2006; 126 (4):869–81. PMID: 16470182
48. Mitsui H, Suarez-Farinas M, Gulati N, Shah KR, Cannizzaro MV, Coats I, et al. Gene expression profiling of the leading edge of cutaneous squamous cell carcinoma: IL-24-driven MMP-7. The Journal of investigative dermatology. 2014; 134(5):1418–27. doi: 10.1038/jid.2013.494 PMID: 24270662
49. Ghavami S, Eshragi M, Ande SR, Chazin WJ, Klonisch T, Halayko AJ, et al. S100A8/A9 induces autophagy and apoptosis via ROS-mediated cross-talk between mitochondria and lysosomes that involves BNIP3. Cell Res. 2009.
50. Ghavami S, Kerkhoff C, Chazin WJ, Kadkhoda K, Xiao W, Zuse A, et al. S100A8/9 induces cell death via a novel, RAGE-independent pathway that involves selective release of Smac/DIABLO and Omi/HtrA2. Biochimica et biophysica acta. 2008; 1783(2):297–311. PMID: 18060880
51. Nukui T, Ehama R, Sakaguchi M, Sonegawa H, Katagiri C, Hibino T, et al. S100A8/A9, a key mediator for positive feedback growth stimulation of normal human keratinocytes. J Cell Biochem. 2008; 104 (2):453–64. PMID: 18044712
52. Ichikawa M, Williams R, Wang L, Vogl T, Srikrishna G. S100A8/A9 activate key genes and pathways in colon tumor progression. Mol Cancer Res. 2011; 9(2):133–48. doi: 10.1158/1541-7786.MCR-10-0394 PMID: 21228116
53. Junttila MR, Li SP, Westermarck J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J. 2008; 22(4):954–65. PMID: 18039929
54. Eue I, Sorg C. Arachidonic acid specifically regulates binding of S100A8/9, a heterodimer complex of the S100 class of calcium binding proteins, to human microvascular endothelial cells. Atherosclerosis. 2001; 154(2):505–8. PMID: 11263412
55. Kerkhoff C, Vogl T, Nacken W, Sopalla C, Sorg C. Zinc binding reverses the calcium-induced arachidonic acid-binding capacity of the S100A8/A9 protein complex. FEBS Lett. 1999; 460(1):134–8. PMID: 10571075
56. Vandal K, Rouleau P, Boivin A, Ryckman C, Talbot M, Tessier PA. Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. J Immunol. 2003; 171(5):2602–9. PMID: 12928412
57. van Lent PL, Grevers L, Blom AB, Sloetjes A, Mort JS, Vogl T, et al. Myeloid-related proteins S100A8/S100A9 regulate joint inflammation and cartilage destruction during antigen-induced arthritis. Annals of the rheumatic diseases. 2008; 67(12):1750–8. PMID: 18055478
58. Qin F, Song Y, Li Z, Zhao L, Zhang Y, Geng L. S100A8/A9 Induces Apoptosis and Inhibits Metastasis of CasKi Human Cervical Cancer Cells. Pathol Oncol Res. 2009.
59. Bluth MJ, Zaba LC, Moussai D, Suarez-Farinas M, Kaporis H, Fan L, et al. Myeloid dendritic cells from human cutaneous squamous cell carcinoma are poor stimulators of T-cell proliferation. The Journal of investigative dermatology. 2009; 129(10):2451–62. doi: 10.1038/jid.2009.96 PMID: 19387481
60. Brooks YS, Ostano P, Jo SH, Dai J, Getsios S, Dziunycz P, et al. Multifactorial ERbeta and NOTCH1 control of squamous differentiation and cancer. J Clin Invest. 2014; 124(5):2260–76. doi: 10.1172/JCI72718 PMID: 24743148
61. Dziunycz PJ, Lefort K, Wu X, Freiberger SN, Neu J, Djerbi N, et al. The oncogene ATF3 is potentiated by cyclosporine A and ultraviolet light A. The Journal of investigative dermatology. 2014; 134(7):1998–2004. doi: 10.1038/jid.2014.77 PMID: 24509533
62. Restivo G, Nguyen BC, Dziunycz P, Ristorcelli E, Ryan RJ, Ozuysal OY, et al. IRF6 is a mediator of Notch pro-differentiation and tumour suppressive function in keratinocytes. The EMBO journal. 2011; 30(22):4571–85. doi: 10.1038/emboj.2011.325 PMID: 21909072

63. Dziunycz P, Hofbauer GF. Immune phenotype of peripheral blood cells and skin squamous cell carcinoma in organ transplant recipients. Expert review of clinical immunology. 2010; 6(3):359–62. doi: 10.1586/eci.10.21 PMID: 20441422

64. Wu X, Nguyen BC, Dziunycz P, Chang S, Brooks Y, Lefort K, et al. Opposing roles for calcineurin and ATF3 in squamous skin cancer. Nature. 2010; 465(7296):368–72. doi: 10.1038/nature08996 PMID: 20485437

65. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008; 3(6):1101–8. PMID: 18546601

66. Hunter MJ, Chazin WJ. High level expression and dimer characterization of the S100 EF-hand proteins, migration inhibitory factor-related proteins 8 and 14. J Biol Chem. 1998; 273(20):12427–35. PMID: 9575199

2.1. Supplementary data

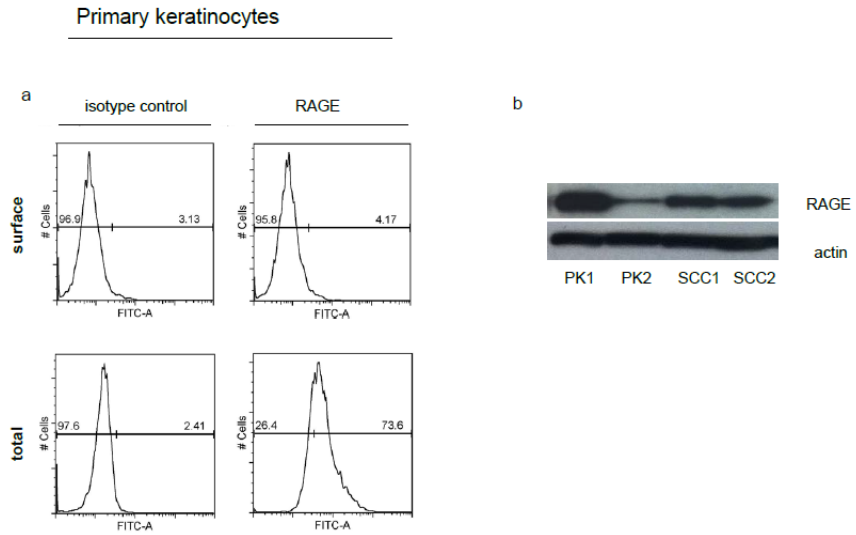
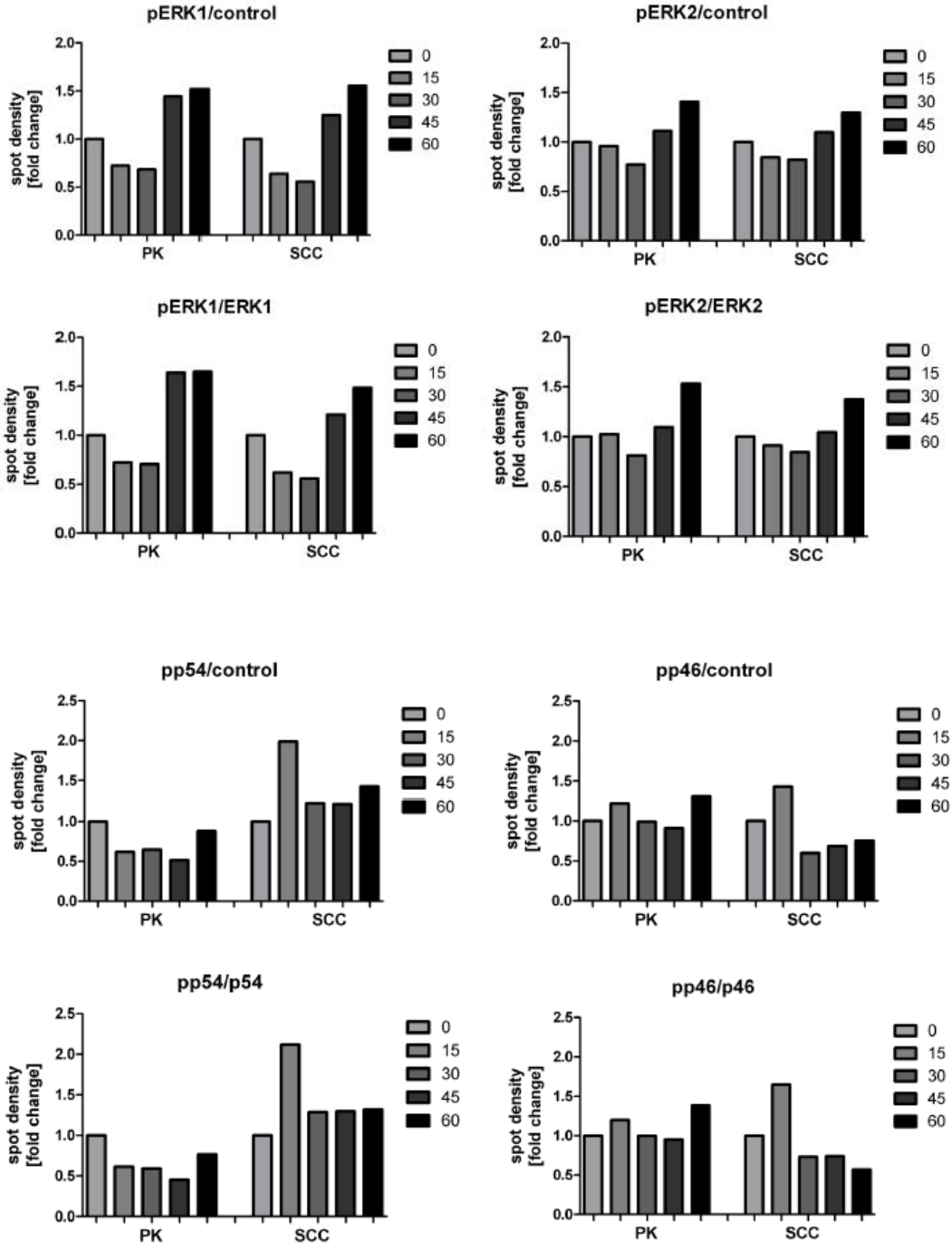


Fig.1. RAGE expression in normal and SCC-derived keratinocytes. a) Surface and total expression of RAGE in normal keratinocytes. Surface RAGE expression was analyzed by FACS using specific anti RAGE antibody and FITC-conjugated secondary antibody. For intracellular staining cells were permeabilized (4%PFA) and blocked (1% BSA) prior staining with the specific RAGE antibody. As a control isotype control antibody was used. b) RAGE expression in normal and SCC derived keratinocytes. Cell lysates from primary normal and SCC keratinocytes were set to SDS PAGE electrophoresis and western blotting was performed using specific primary anti RAGE and secondary HRP-conjugated antibodies. As a loading control additional staining against actin was performed using specific anti actin antibody.


pERK1/control
spot density [fold change]
2.0
1.5
1.0
0.5
0.0
PK
SCC
0
15
30
45
60
pERK2/control
spot density [fold change]
2.0
1.5
1.0
0.5
0.0
PK
SCC
0
15
30
45
60
pERK1/ERK1
spot density [fold change]
2.0
1.5
1.0
0.5
0.0
PK
SCC
0
15
30
45
60
pERK2/ERK2
spot density [fold change]
2.0
1.5
1.0
0.5
0.0
PK
SCC
0
15
30
45
60
pp54/control
spot density [fold change]
2.5
2.0
1.5
1.0
0.5
0.0
PK
SCC
0
15
30
45
60
pp46/control
spot density [fold change]
2.5
2.0
1.5
1.0
0.5
0.0
PK
SCC
0
15
30
45
60
pp54/p54
spot density [fold change]
2.5
2.0
1.5
1.0
0.5
0.0
PK
SCC
0
15
30
45
60
pp46/p46
spot density [fold change]
2.5
2.0
1.5
1.0
0.5
0.0
PK
SCC
0
15
30
45
60

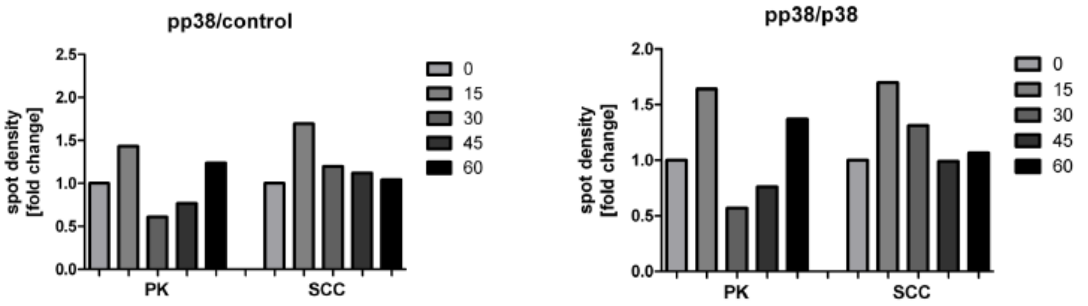


Fig.2. Densitometrical quantification of phosphorylated ERK, JNK and p38 proteins versus control. a) Spot density-based quantification of pERK1/2 versus ERK1/2 and versus loading control (actin). b) Spot density-based quantification of pSAPK/JNK (pp54/p46) versus SAPK/JNK and versus loading control (actin). c) Spot density-based quantification of pp38 versus p38 and versus loading control (actin).

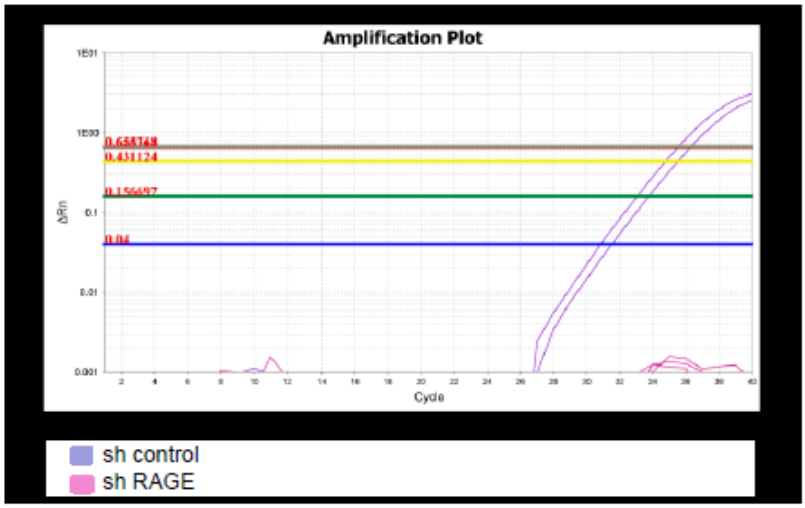


Fig.3. RAGE expression in normal keratinocytes after RAGE knockdown by lentiviral shRNA. The RAGE expression after knockdown was analyzed on transcriptional level by qPCR using specific primers for RAGE and compared to sh control.

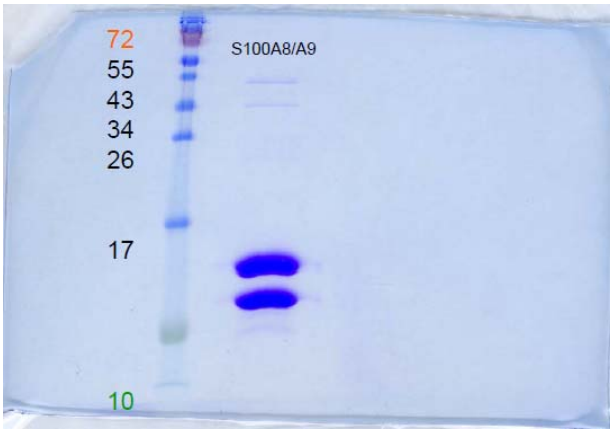


Fig.4. S100A8/A9 protein purification. S100A8/A9 has been extracted from granulocytes of buffy coats. The purity, quantity and quantity of the extracted S100A8/A9 were analyzed by Coomasie blue staining after SDS gel electrophoresis.

3. TLR4 as a direct negative regulator of keratinocyte proliferation

Toll-like receptor 4 (TLR4) is known to play a role in the recognition of gram-negative pathogens and its downstream signaling triggers a number of immune responses. TLR4 is expressed on healthy cells, among them keratinocytes, but also on various tumor cells. While some studies reveal a tumor promoting effect of TLR4, others suggest the opposite, leaving the role of TLR4 in tumor development under controversial discussion. In the current project, we found TLR4 to negatively regulate keratinocyte proliferation. *In vitro* experiments as well as xenograft studies in mice with injection of tumor cells overexpressing TLR4 and TLR4-knock-down keratinocytes clearly showed the impact of TLR4 on cell- and tumor growth.

Contribution to the manuscript:
I contributed to this project by isolating primary cells from tissue, by performing differentiation experiments and proliferation assays as well as by helping injecting and monitoring the mice. Furthermore, I participated in experiment planning, discussion and manuscript preparation.

TLR4 as a negative regulator of keratinocyte proliferation

Iotzova-Weiss, Guergana[1*]; Freiberger, Sandra N.[1]; Johansen, Pål[1]; Levesque, Mitchell P.[1]; Kamarachev, Jivko[1]; Guenova-Hötzenecker, Emmanuella [1]; Dziunycz, Piotr J.[1]; Roux, Guillaume [2]; Liu, Qinxiu[1]; Neu, Johannes [1]; Hofbauer, Günther F. L.[1]

[1] Department of Dermatology, University Hospital Zurich, Zurich, Switzerland
[2] Department of Dermatology, CHUV, Lausanne, Switzerland

* Corresponding author
Guergana Iotzova-Weiss, PhD, Department of Dermatology, University Hospital Zurich, Gloriastrasse 31, 8091 Zürich, Switzerland, Phone +41 44 255 5528, Guergana.Iotzova@usz.ch

Key words
squamous cell carcinoma of the skin, Toll-like receptor 4, keratinocyte proliferation, tumor growth

Abbreviations
TLR4: Toll-like receptor 4
SCC: Squamous Cell Carcinoma
ERK: Extracellular-signal Regulated Kinases
JNK: cJUN-N-terminal kinase
MyD88: Myeloid differentiation primary response 88
CDH13: H cadherin 13
ATF3: Cyclin-AMP-dependent transcription factor 3
CXCL-1: Chemokine (C-X-C motif) ligand 1
TFPI: Tissue factor pathway inhibitor

3.1. Abstract

In the present study we investigate the role of TLR4 as a negative regulator of keratinocyte proliferation. TLR4 is an innate immune receptor with expression in normal human skin and keratinocytes as well as in cutaneous squamous cell carcinoma (SCC). We present here that the expression of TLR4 increased with the differentiation of cultured keratinocytes in a passage-dependent manner or under calcium-rich conditions. Moreover, the down-regulation of TLR4 by specific knockdown increased the proliferation of primary normal, SCC-derived and HaCaT keratinocytes in vitro. In addition, subcutaneously injected HaCaT keratinocytes with shTLR4 formed growing tumors in nude mice. In contrast, we observed lower proliferation and increased migration in vitro of the SCC13 cell line stably overexpressing TLR4 in comparison to SCC13 TLR4-negative cells. In vivo, SCC13 TLR4-overexpressing tumors showed delayed growth in comparison to TLR4-negative tumors. The overexpression of TLR4 in SCC13 tumor cells was followed by phosphorylation of ERK1/2 and JNK and increased expression of MyD88. In gene expression arrays, the overexpression of TLR4 in tumor cells correlated with gene expression of CDH13, CXCL-1, ATF-3, IL-6, and TFPI. In summary, TLR4 negatively regulates keratinocyte proliferation in SCC and normal keratinocytes.

3.2. Introduction

The keratinocyte cell cycle is determined by proliferation and terminal differentiation, two processes that control and maintain normal skin homeostasis. The basal keratinocyte layer is characterized by highly proliferative keratinocytes, expressing the differentiation markers K5 and K14. Well differentiated keratinocytes sustain the spinous, granular and corneal layers, have decreased proliferation potential and are characterized by the expression of K1, K10, involucrin, loricrin and filaggrin (Kypriotou, Huber, & Hohl, 2012; Kalinin, Marekov, & Steinert, 2001; Koster & Roop, 2007). The signaling cascades regulating the process of keratinocyte differentiation are dependent on the crosstalk between the different epidermal layers. Among the signaling pathways involved in keratinocyte differentiation are Notch (Massi & Panelos, 2012; Moriyama et al., 2008; Nguyen et al., 2006); Wnt (Lim & Nusse, 2013) and p63 (Barbieri & Pietenpol, 2006; Koster & Roop, 2007). The dysregulation of these signaling pathways is observed in skin diseases such as SCC and psoriasis (Dotto, 2011; Dotto, 2008; Restivo et al., 2011; Okuyama, Tagami, & Aiba, 2008).

Toll like receptor 4 (TLR4) is a pattern recognition receptor and a key component of the innate immune system. TLR4 is moreover expressed in skin and cultured keratinocytes (Begon et al., 2007; Lebre et al., 2007). Of relevance for keratinocyte biology, it is reported that the adaptor protein TRIP (TRAF-interacting protein) regulates keratinocyte proliferation and differentiation (Almeida, Ryser, Obarzanek-Fojt, Hohl, & Huber, 2011). The role of TLR4 is investigated in skin diseases such as dermatitis and psoriasis (Panzer, Blobel, Folster-Holst, & Proksch, 2014); SCC (Muehleisen et al., 2012) and melanoma (Bald et al., 2014), as well as in skin wound healing (Suga et al., 2014; Loryman & Mansbridge, 2008). TLR4 signaling is reported to promote development of SCC in a MyD88-dependent manner and to be required for the recruitment of inflammatory cells during carcinogenesis (Mittal et al., 2010). The development of epithelial tumors is also described through the interaction of TLR4 with HMGB-1 in the extracellular skin fluid, causing the activation of NF-κB in keratinocytes. Thus, TLR4 mediates between inflammation and epithelial tumor development (Weng et al., 2013). TLR4 is also reported to play a role in the prevention of chemically induced carcinogenesis through the activation of T cells (Yusuf et al., 2008). The role of TLR4 in supporting cell growth or inducing apoptotic signals is presented mostly through the activation of other cell populations. However, a relation between TLR4 expression on keratinocytes and their proliferation has not been investigated by now. In the present study we propose a novel role for TLR4 as a regulator of keratinocyte proliferation.

3.3. Materials and methods

The use of clinically indicated biopsy material for the study was approved by the ethical committee of the Canton of Zürich, Switzerland. Healthy skin was obtained from patients at plastic surgery following informed consent as approved by the ethical committee of the Canton of Zürich, Switzerland, and described previously (Brooks et al., 2014; Dziunycz et al., 2014; Restivo et al., 2011; Wu et al., 2010).

Immunohistochemistry (IHC)

TLR4 expression was analyzed in formalin-fixed paraffin-embedded skin samples obtained from the archives of the Dermatology Department of University Hospital Zurich (approval and agreement by the patients is documented). The expression was tested in normal skin and in moderately and well differentiated SCC skin derived from

organ transplant recipients and immunocompetent patients. The diagnosis and the classification of the SCC samples were performed by a board of certified dermatopathologists.
The staining was performed using mouse anti-human TLR4 monoclonal antibody (HTA125, Abcam) following the manufacturers` protocol.

Cell lines
The HaCaT cell line was obtained from Prof. Petra Boukamp, DKFZ, Heidelberg, Germany. The SCC13 cell line was generated by Prof. Rheinwald *(Rheinwald & Beckett, 1981)*.

Generation of primary keratinocyte cultures derived from healthy individuals and SCC patients
Primary normal and SCC-derived keratinocytes were generated in-house from normal skin (abdominoplastic reconstructive surgery) and biopsies from patients' SCC samples. Single keratinocytes were isolated from 3-4mm punch biopsies following the standard protocol for generation of primary keratinocyte cultures (CELLnTEC, Bern, Switzerland). For sufficient separation of the epidermis and dermis the punch biopsies were incubated overnight at 4ºC in keratinocyte selection medium (Progenitor Cell Targeted (PCT) epidermal keratinocyte medium CnT07) (CELLnTec) with Dispase II (Roche). Afterwards the epidermal part of the skin was incubated in trypsin at room temperature for 30 min to insure efficient isolation of single keratinocytes. Freshly isolated cells were washed with medium and then transferred into CnT07 medium for a continuous incubation (37°C, 5%CO2).

Immunoblotting
Differentiation of normal primary keratinocytes
Normal primary keratinocytes were grown in 6cm dishes in 50% and full confluence or under Ca conditions (1.5mM $CaCl_2$) in CnT07 keratinocyte medium (CELLnTEC, Bern, Switzerland). Cells were lysed in RIPA buffer (conventional protocol). The lysates were collected and subjected to SDS-PAGE, followed by immunoblotting using specific antibodies against human TLR4 (H-80, sc10741) involucrin (sc-21748), filaggrin (sc-66192) and actin (sc-47778), all from Santa Cruz Biotechnology.

LPS stimulation of SCC13 TLR4 overexpressing cells

SCC13 TLR4 overexpressing and SCC13 control cells were grown in 6cm dishes in serum free DMEM medium for 24 hours. Afterwards cells were stimulated with ultrapure LPS (10μg/ml, Sigma) for different time intervals (0-60 min). Cells were lysed in RIPA buffer; the lysates were collected after each time interval and subjected to SDS-PAGE, followed by immunoblotting. The phosphorylated and non-phosphorylated form of ERK1/2 and JNK was detected by using specific rabbit polyclonal antibodies (anti-pp42/44 (#9101), anti-p42/44 (#9102); anti-rabbit HRP (#7074), all from Cell Signaling; anti-β-Actin (sc-47778, Santa Cruz Biotechnology, anti-mouseHRP (ab6728, Abcam). The expression of MyD88 (ab2064, Abcam) and IRAK-1 (D51G7, #4504, Cell signalling) upon LPS stimulation was analyzed using the same protein lysates. The secondary HRP-conjugated antibodies were used based on their species specificity to the primary antibodies.

Knockdown study

Primary normal and SCC-derived keratinocytes, as well as HaCaT keratinocytes were infected with lentiviral particles carrying specific shTLR4 consruct (sc- 40260-v) or shCtrl lentiviral particles (sc-108080), following the protocol conditions (Santa Cruz Biotechnology). Positive clones were selected by puromycine selection. The growth of the clones after TLR4 knockdown and controls was analyzed by BrdU proliferation assay.

RNA extraction and qPCR

RNA was extracted using the TRIzol reagent (Invitrogen, Basel, Switzerland) following the protocol provided by the manufacturer. cDNA was synthesized from 1μg total RNA using a Reverse Transcription system kit (Promega). Specific primers for human TLR4 (Microsynth, Switzerland), (fwd 5‘ GCCCTGCGTGGAGGTGGTTC 3‘; rev 5‘ TGAGAAGGGGAGGTTGTCGGGG 3‘) were used. PCRs were performed by ViiA7 ™ Real-Time PCR System (Life Technologies) using FastStart Universal SYBR green Mix (ROX) (Roche).

Overexpression study

SCC13 tumor cells were stably transfected with control (pUNO, Invivogen) and TLR4 expressing plasmid (pUNO-TLR4GFP, Invivogen) following the manufacturer's protocol. For the selection of positive clones the transfected cells were further grown in DMEM medium containing blasticidine (10μg/ml) as a selection marker. The positive clones were visualized based on their GFP signal.

Scratch assay

SCC13 TLR4+ and control cells were treated with Mytomycine C (10 μg/ml) Sigma Aldrich) prior to the scratch assay. The scratch assay was performed on 100% confluent cells using a blue pipette tip for generating a cross region in every well. The number of migrated cells across the marked region was counted and presented as percent of control.

BrdU proliferation assay

Cells were seeded in 96 well plates in serum-free CnT07 or Dulbecco's Modified Eagle Medium, DMEM in a cell density of $4x10^3$/well. Cell proliferation was measured using BrdU proliferation assay (Millipore) according to the manufacturer's instruction. The percentage of cell proliferation was calculated using the equation: (mean OD of treated cells/mean OD of control cells) X 100.

Gene expression array

The gene expression was analyzed in SCC13 TLR4 and control cells in tetraplicates. RNA was isolated and cDNA generated by reverse transcription. The SurePrint G3 Human Gene Expression 8x60K (Agilent) with 50599 biological features was used to analyze the samples. Differentially expressed genes were selected to be relevant if the absolute log-fold change was more than 2 and significant when FDR adjusted p-value was less than 0.05. The expression of these genes was validated by PCR.

Mice

The *in vivo* growth of HaCaT and SCC13 cell lines was analyzed in athymic nude mice (Hsd: *Athymic Nude-Fox1^{nu}/Foxn1^{+}*, Harlan).

Growth of shTLR4 and csh HaCaT in nude mice

HaCaT cells stably transfected with shTLR4 or control sh were injected into nude mice subcutaneously ($4x10^6$ cells/mouse; 6 mice per group). First measurement of the tumor size was performed 2 weeks after injection and approximately every 2 weeks further on. The tumor size was measured by caliper, and the tumor volume was calculated using (height X ($width^2$))/100 formula, where width was the shorter distance. The differential growth between shTLR4 and control sh tumors was analyzed statistically for every time point and end day by t-test (p=0.0002).

Growth of SCC13 TLR4 overexpressing cells in nude mice

SCC13 TLR4 overexpressing and control cells were injected subcutaneously in nude mice ($4x10^6$ cells/mouse) in total of 22 animals divided into 3 groups. First measurement of the tumor size was performed 1 week after injection and approximately every week further on. The tumor size was measured by caliper, and the tumor volume was calculated using the (height X ($width^2$))/100 formula, where width was the shorter distance. The animals were sacrificed, when the tumors reached either a volume of 1000 mm^3 or ulcerated. The differential growth between TLR4 overexpressing and control SCC13 tumors was analyzed based on the tumor volume for all tumors above 100 mm^3 (p=0.0217, non-parametric Mann-Whitney t-test).

The expression of Ki67 proliferation marker in the tumor tissues was analyzed by IHC and the differences in the expression levels between TLR4 overexpressing tumors and control tumors were presented as [%] positive cells in the tumor area.

3.4. Results

Expression of TLR4 in normal and SCC skin

The total TLR4 expression was analyzed in the epidermis of normal and SCC-derived patient samples and visualized by immunohistochemistry (Fig. 1). Thereby we confirmed the expression of TLR4 in normal human skin, as well as in SCC tissue of various patients.

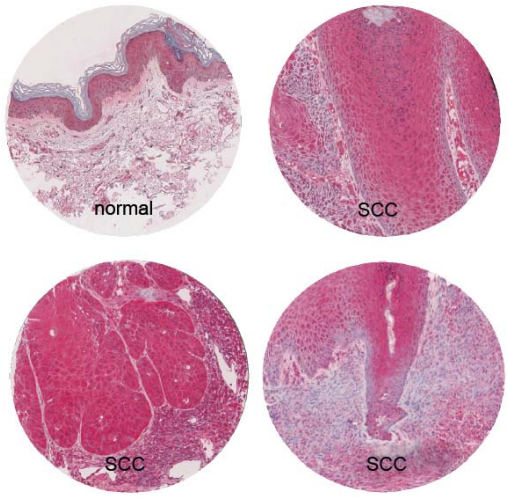


Fig. 1: Immunohistochemical staining of TLR4 in normal human skin and SCC tissue from different patients. SCC = squamous cell carcinoma.

TLR4 expression increases with the differentiation of normal keratinocytes in vitro

TLR4 expression was investigated in growing and differentiating normal primary keratinocytes. Differentiating keratinocytes were characterized by higher involucrin and filaggrin expression and showed increased TLR4 expression (Fig. 2a). Similarly, increased TLR4 and involucrin expression was detected when primary and SCC-derived keratinocytes were driven to differentiation under calcium-rich conditions after 48h and 4 days (Fig. 2b).

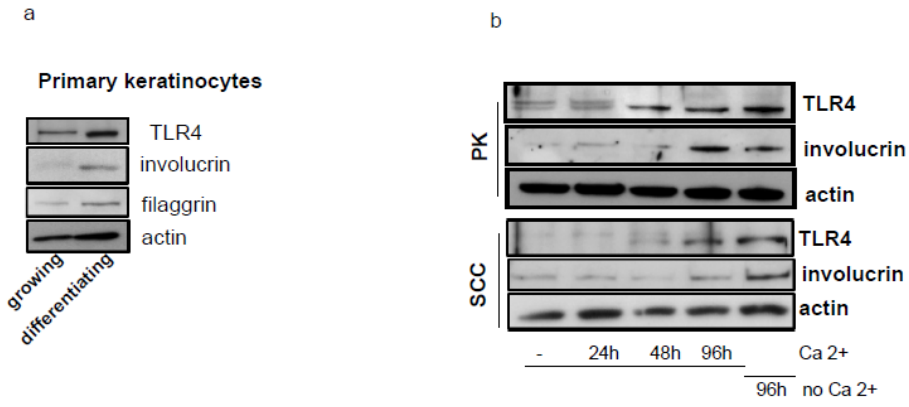


Fig. 2: TLR4 expression increases with differentiation of normal and SCC keratinocytes. a) Protein extracts of growing or differentiated primary keratinocytes analyzed for TLR4 expression. Differentiation of keratinocytes was confirmed by expression of differentiation markers involucrin and filaggrin. Actin served as loading control. b) Protein extracts from keratinocytes and SCC cells, either grown under normal or calcium-rich conditions for 1 – 4 days were analyzed for TLR4 expression. Differentiation was confirmed by expression of the differentiation marker involucrin. Actin served as loading control.

Knockdown of TLR4 by specific lentiviral short-hairpin RNA (shRNA) induces keratinocyte proliferation

Primary normal, SCC-derived and HaCaT keratinocytes were transfected with a specific lentiviral shRNA against TLR4 and a control shRNA. Induction of keratinocyte proliferation after TLR4 knockdown was assessed by BrdU incorporation and representative pictures were taken (Fig. 3a). Primary normal and SCC-derived keratinocytes transfected with a control shRNA showed a delayed proliferation and did not reach confluence due to their short life span. Therefore, a reliable quantification of the TLR4 level by qPCR or immunoblotting in these cells was not technically possible. The successful knockdown was verified by PCR and immunoblotting only in the long-living HaCaT cells (Fig 3b and 3c.).

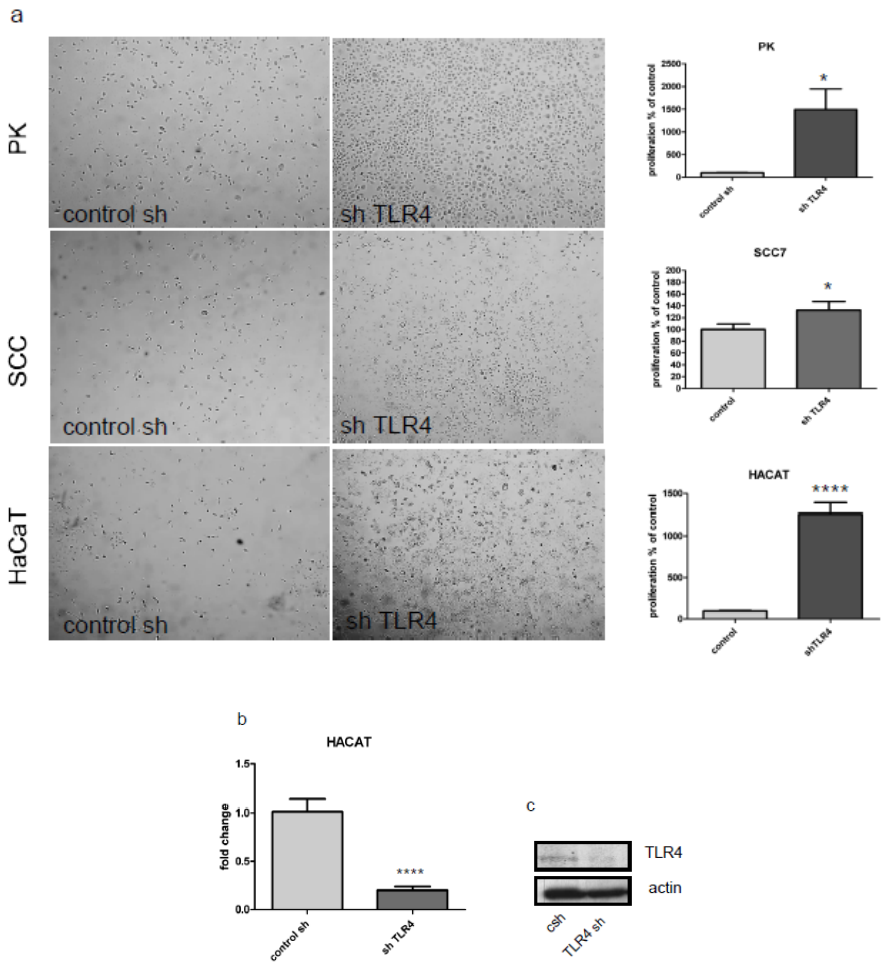


Fig. 3: Knockdown of TLR4 increases proliferation of normal and SCC keratinocytes. a) Cells were infected with lentiviral constructs and cultured under puromycin selection. Proliferation was assessed by BrdU

incorporation and representative pictures were taken. b) Confirmation of TLR4 knockdown in HaCaT cells by qPCR. c) Confirmation of TLR4 knockdown in HaCaT cells by western blotting. All graphs represents mean +/- sd. Significance was analyzed by student's t-test (*= p<0.05, ****= p<0.0001).

Growth of shTLR4 HaCaT keratinocytes in nude mice

HaCaT shTLR4 keratinocytes were injected subcutaneously in nude mice and the growth of the tumors was investigated at different time points over 3 months. Mice injected with TLR4 knockdown cells developed progressively growing tumors in comparison to mice injected with control cells (difference at end point, p=0.0002, t-test, Fig. 4a). Only 1 out of 6 control tumors was still detectable 102 days after injection. This control tumor served as a basis for comparison of the TLR4 expression level (Fig. 4b).

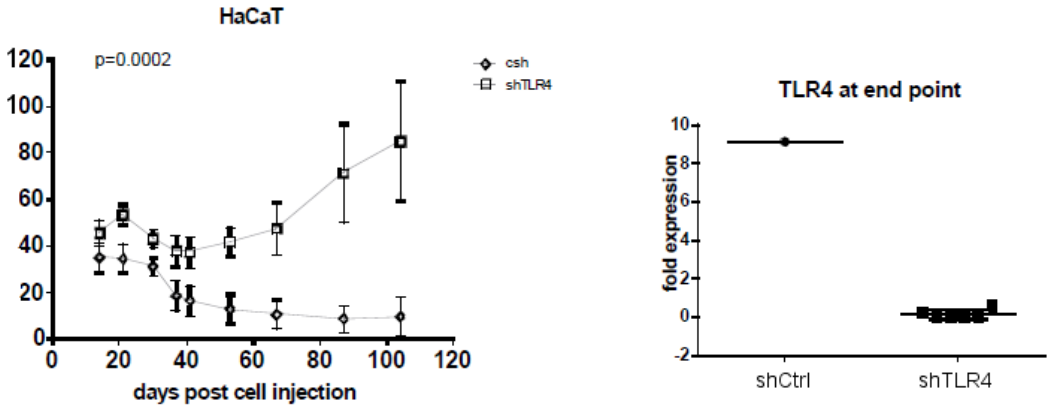


Fig. 4: Knockdown of TLR4 increases tumor growth *in vivo*. a) TLR4 was knocked down in HaCaT cells using lentiviral constructs. Cells were then injected into nude mice and tumor growth was measured over time. b) RNA was extracted from tumors after termination of the experiment and subject to qPCR to confirm TLR4 knockdown in tumor cells.

SCC13 TLR4-overexpressing cells show a delay in proliferation and higher migratory activity in vitro

SCC13 tumor cells were stably transfected with TLR4-expressing and control plasmid and maintained in culture under selection conditions (Blasticidine, 10µg/ml). We detected three populations of GFP-TLR4 expressing cells with differential intensity of GFP expression (Suppl. Fig. 1). Cells with highest GFP signal were sorted by FACS and used for further investigations. Representative pictures are shown (Fig. 5a). TLR4-overexpressing cells showed lower proliferation compared to control as detected by BrdU incorporation (Fig. 5b). Using conventional scratch assay we detected increased migration of TLR4-overexpressing SCC13 cells in comparison to control cells (Fig. 5c).

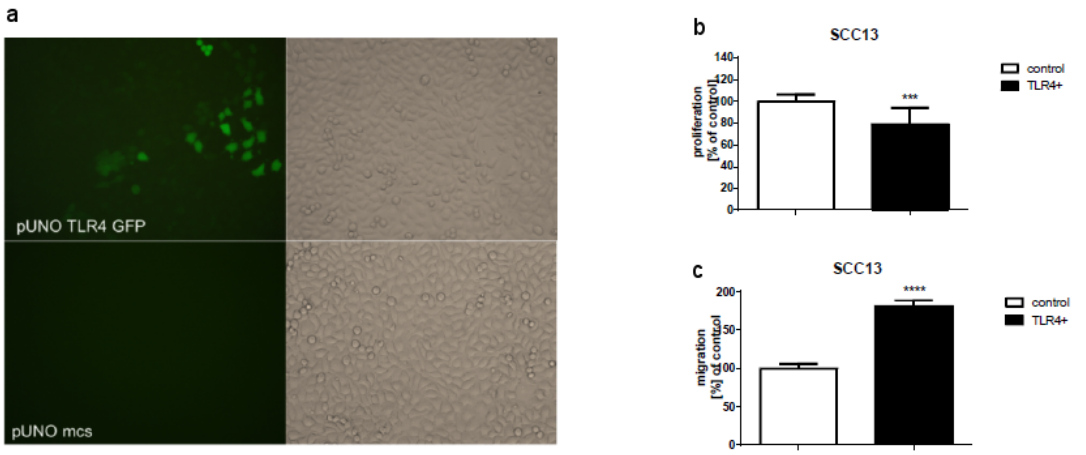


Fig. 5: TLR4 overexpression decreases proliferation and increases migratory activity of SCC13 cells. a) Representative pictures of GFP-TLR4-overexpressing cells and cells transfected with control plasmid. b) SCC13 cells were stably transfected with TLR4 and proliferation was assessed by BrdU incorporation. c) SCC13 cells were stably transfected with TLR4 and migration was assessed by scratch assay. All graphs represent mean +/- sd of three independent experiments. Significance of the results was analyzed by student's t-test (***= $p<0.001$, ****= $p<0.0001$).

Growth of SCC13 TLR4 overexpressing cells in vivo

SCC13 TLR4-overexpressing cells (TLR4+) and SCC13 control cells were injected subcutaneously into nude mice. Tumor growth was analyzed based on tumor volume at different time points starting at 1 week after injection. SCC13 TLR4+ tumors showed slower growth in comparison to control tumors (mean tumor volume: 350 mm^3 for control and 250 mm^3 for TLR4-overexpressing tumors; $p=0.0217$, Man-Whitney, Fig. 6a). TLR4-overexpressing tumors showed a lower expression of Ki67 in comparison to control tumors (Fig. 6b) which reflects the slower growth of these tumors. The tumors were still overexpressing TLR4 on the day of experiment termination as detected by PCR (Fig. 6c). The expression of keratinocyte differentiation markers was analyzed by qPCR. We detected a variable expression profile of involucrin, filaggrin and K1 within the tumors (data not shown). We did not detect any tumor cells in the surrounding lymph nodes, lung or liver of the mice on the day of experiment termination (data not shown). Extended time points for investigation of metastatic potential were not possible, as fast tumor growth combined with tumor ulceration required us to sacrifice animals as by the law.

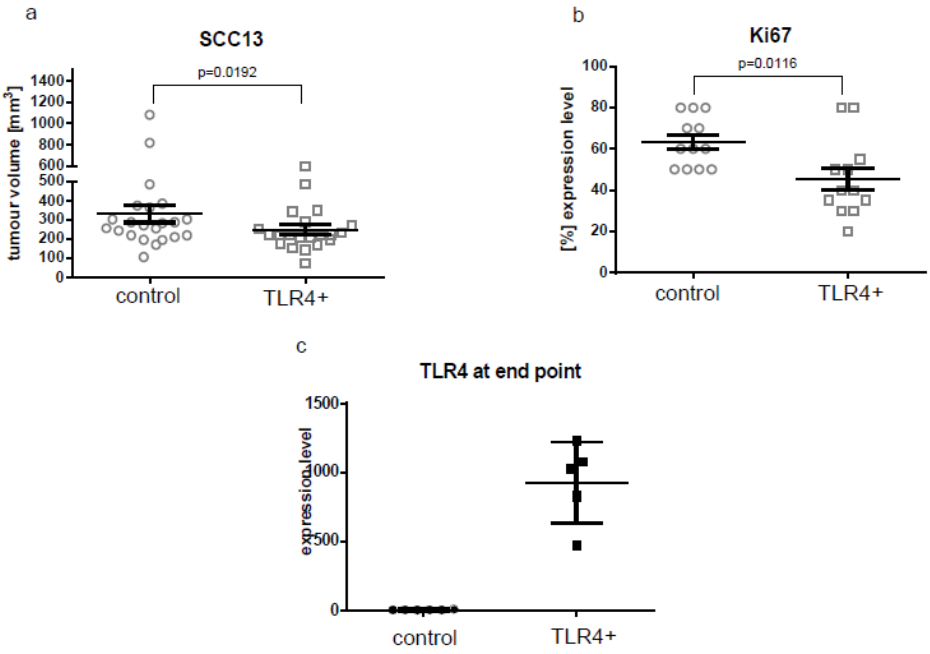


Fig. 6: Overexpression of TLR4 delays tumor growth *in vivo*. a) TLR4-overerexpressing or control cells were injected into nude mice. Tumor growth was measured over time. b) Tumor tissue was stained for the proliferation marker Ki67. Ki67-positive cells were counted in the tumor area and converted to % of positive cells. c) RNA from tumors was extracted after termination of the experiment to confirm TLR4 overexpression by qPCR.

Functional relevance of TLR4 in SCC13 TLR4 expressing cells

Phosphorylation of ERK and JNK in TLR4-overexpressing SCC13 cells

The functional relevance of TLR4 was tested by LPS stimulation. We analyzed the phosphorylation of ERK and JNK at different time points after LPS stimulation (Fig. 7a). We detected increased basal phosphorylation of JNK in unstimulated TLR4-overexpressing SCC13 cells in comparison to control cells. The increased phosphorylation was still detectable 15 min after LPS stimulation and decreased further in the course of LPS treatment. Similarly, increased phosphorylation of ERK in TLR4-overexpressing cells was observed in LPS-free conditions, which in contrast to JNK was inducible in presence of LPS. We did not detect any alteration of phosphorylation of IRAK1 (data not shown).

Overexpression of TLR4 leads to differential gene expression in SCC13 cells

Differential gene expression profile between TLR4-overexpressing and control SCC13 cells was investigated by cDNA microarray analysis. Upregulated and downregulated

genes were selected based on their statistically significant alteration in the expression level (Fig. 7b). Within the upregulated genes were CDH13, CXCL-1 and ATF3 and within the downregulated genes were IL-6 and TFPI. IL6 downregulation and ATF3 upregulation were confirmed on protein level by ELISA (IL6) and western blotting (ATF3) (Fig. 7b and 7c).

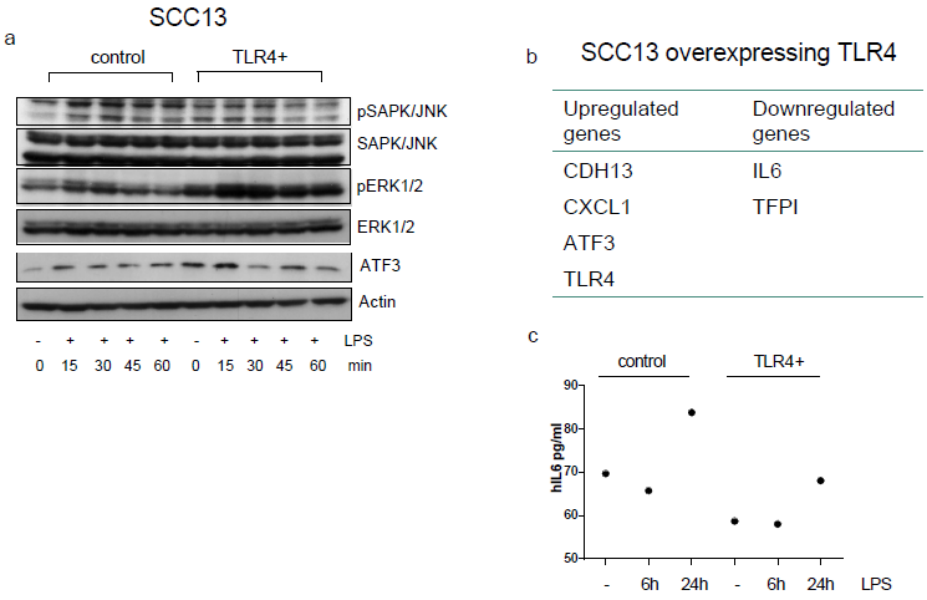


Fig. 7: TLR4 has a functional relevance in SCC13 cells following overexpression. a) TLR4-overexpressing and control cells were treated with LPS. Phosphorylation status of JNK and ERK as well as ATF3 protein expression was analyzed in total protein extracts by western blotting. Actin served as loading control. b) mRNA of TLR4-overexpressing cells was analyzed by gene expression array. Top up- and down-regualted genes are displayed. c) IL6 protein level of TLR4-overexpressing and control cells was analyzed by ELISA after LPS stimulation.

3.5. Discussion

Within this study we investigate the role of TLR4 as a regulator of keratinocyte proliferation. We analyzed the expression of TLR4 in normal skin and SCC tissue, where we detected immunoreactivity for TLR4 in all samples, thereby confirming their expression in normal and SCC tissue.

Furthermore, we investigated the expression of TLR4 in the context of differentiation in vitro. We found a correlation between the level of TLR4 and keratinocyte differentiation in normal and SCC keratinocytes. A relation between passage growth and TLR4 expression has been previously observed, however in HaCaT cell line (Pivarcsi & Homey, 2005).

A functional relation between TLR4 and keratinocyte proliferation and differentiation has not been investigated by now. In our study we present that knockdown of TLR4 induces proliferation of keratinocytes in vitro and in vivo. Hyperproliferation of keratinocytes is observed mostly in inflammatory skin diseases as psoriasis (Nestle et al., 2005; Pasparakis, Haase, & Nestle, 2014). TLR4 is expressed in psoriatic skin (Baker, Ovigne, Powles, Corcoran, & Fry, 2003; Panzer et al., 2014), however, a direct regulation of proliferation by TLR4 in keratinocytes has not been discussed. Studies in TLR4-deficient mice and in vitro studies revealed involvement of TLR4 in skin wound healing (Suga et al., 2014; Chen, Guo, Ranzer, & DiPietro, 2013). In contrast to our results the authors describe activation of TLR4 signaling upon immune stimuli, which induces keratinocyte proliferation. A direct involvement, however, of TLR4 expressed on keratinocytes as a negative regulator of keratinocyte proliferation is not discussed.

Our study suggests that TLR4 is a regulator of proliferation and growth of SCC tumors as well. We show that TLR4 overexpression decreases proliferation and induces migration of SCC13 cells in vitro and delays growth of SCC13 tumors in vivo. SCC13 cells are suitable model for investigation of TLR4-dependent proliferation, however, differentiation of SCC13 cells based on TLR4 expression proved difficult to ascertain due to an insufficient Notch signaling pathway in these cells (Lambertini, Pantano, & Dotto, 2010; Lefort & Dotto, 2004).

Generally, SCC13 cells are known for their high metastatic potential (Choi et al., 2014; Toll et al., 2013). We, however, did not detect any tumor cells in lymph nodes or other organs of the treated animals, possibly due to early termination of the experiments based on ulceration of the primary tumor, forcing an early sacrifice of animals.

Human material and in vivo models in rodents have been studied for the involvement of TLR4 in tumor progression, although the focus of these projects was mainly on the involvement of the innate immune system (Szczepanski et al., 2009; Mittal et al., 2010; Yusuf et al., 2008; Ilmarinen et al., 2014; Takazawa et al., 2014; Bald et al., 2014). Studies in TLR4-deficient mice revealed that TLR4 has a supportive role in the development of chemically induced skin cancer through immune response (Yusuf et al., 2008; Mittal et al., 2010). While these mice are globally deficient for TLR4 with a big impact on innate immunity to be expected, tumor cell cultures originating directly from the DMBA-induced skin tumors in TLR4-deficient mice, however, show an increased proliferative

capacity in comparison to tumors of control mice in the absence of immune cells. This supports our data concerning TLR4 expression, keratinocytes proliferation and tumor growth.

We observed specific phosphorylation changes of pERK and pJNK dependent on the TLR4 levels in tumor keratinocytes. The phosphorylation pattern of ERK and JNK after LPS stimulation in TLR4 overexpressing cells confirms the functionality of TLR4 in our model. Based on our results we suggest a relationship of ERK and JNK pathways to TLR4 overexpression, possibly contributing to the balance of proliferation and differentiation.

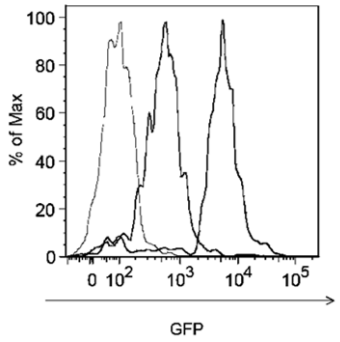
Furthermore, using gene expression analysis in SCC13 TLR4-overexpressing cells we selected and validated genes reported to be involved in proliferation and migration of malignant cells. Within these upregulated genes were CDH13 (Cadherin H), known to participate as a modulator of proliferation and migration in melanoma, SCC and BCC (Kuphal et al., 2009; Takeuchi, Liang, Matsuyoshi, et al., 2002; Takeuchi, Liang, & Ohtsuki, 2002); CXCL-1, involved in the control of epithelial-mesenchymal interaction in normal or malignant epithelial cells (Kolar et al., 2012); and ATF3 reported as transcription factor in epithelial cancer (Choi et al., 2014; Dziunycz et al., 2014; Wu et al., 2010). Among the downregulated genes was IL6, known to be involved in the metastatic and invasion properties of melanoma, SCC of the skin and head and neck SCC (Ivanov, Partridge, Huang, & Hei, 2011; Prasad et al., 2014). IL6 expression is known to be negatively regulated by ATF3 (Calton CM et al, 2013; Gilchrist M et al., 2007). This correlates with our results showing that TLR4-overexpressing cells have increased ATF3 expression and decreased IL6 secretion, which confirms the relation between TLR4, ATF3 and IL6. Within the downregulated genes was also TFPI, involved in the formation of matrix-rich vascular-like networks in melanoma (Ruf, 2003; Ruf et al., 2003; Kageshita et al., 2002) and a diagnostic marker of SCC (Lai et al., 2014).

Without further work-up of these individual genes, which have all been described in cancer models and tissue, we currently interpret their dependence on TLR4 expression in our data as support for the suggested role of TLR4 in tumor growth.

Altogether we assume that TLR4 impacts keratinocyte biology as a regulator of proliferation in normal and tumor keratinocytes and also in the migration of tumor cells. Thus TLR4 as negative regulator of keratinocyte proliferation may associate with the progression of SCC of the skin. A better understanding of the regulatory role for TLR4

will be the basis for a later use in a therapeutic setting to impair keratinocyte proliferation such as in squamous cell carcinoma of the skin and to induce keratinocyte proliferation such as in wound healing.

3.6. Supplementary data



Suppl.Fig. 1: FACS analysis of GFP-TLR4-overexpressing SCC13 cells. Three different populations with differing signal intensity were detected.

3.7. References

Almeida, S., Ryser, S., Obarzanek-Fojt, M., Hohl, D., & Huber, M. (2011). The TRAF-interacting protein (TRIP) is a regulator of keratinocyte proliferation. J Invest Dermatol, 131(2), 349-357. doi: 10.1038/jid.2010.329

Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Bare, O., Auron, P. E., . . . Calderwood, S. K. (2002). Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem, 277(17), 15028-15034. doi: 10.1074/jbc.M200497200

Baker, B. S., Ovigne, J. M., Powles, A. V., Corcoran, S., & Fry, L. (2003). Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. Br J Dermatol, 148(4), 670-679.

Bald, T., Quast, T., Landsberg, J., Rogava, M., Glodde, N., Lopez-Ramos, D., . . . Tuting, T. (2014). Ultraviolet-radiation-induced inflammation promotes angiotropism and metastasis in melanoma. Nature, 507(7490), 109-113. doi: 10.1038/nature13111

Barbieri, C. E., & Pietenpol, J. A. (2006). p63 and epithelial biology. Exp Cell Res, 312(6), 695-706. doi: 10.1016/j.yexcr.2005.11.028

Begon, E., Michel, L., Flageul, B., Beaudoin, I., Jean-Louis, F., Bachelez, H., . . . Musette, P. (2007). Expression, subcellular localization and cytokinic modulation of Toll-like receptors (TLRs) in normal human keratinocytes: TLR2 up-regulation in psoriatic skin. Eur J Dermatol, 17(6), 497-506. doi: 10.1684/ejd.2007.0264

Brooks, Y. S., Ostano, P., Jo, S. H., Dai, J., Getsios, S., Dziunycz, P., . . . Dotto, G. P. (2014). Multifactorial ERbeta and NOTCH1 control of squamous differentiation and cancer. J Clin Invest, 124(5), 2260-2276. doi: 10.1172/JCI72718

Bryant, C. E., Spring, D. R., Gangloff, M., & Gay, N. J. (2010). The molecular basis of the host response to lipopolysaccharide. Nat Rev Microbiol, 8(1), 8-14. doi: 10.1038/nrmicro2266

Bryant, C. E., Symmons, M., & Gay, N. J. (2014). Toll-like receptor signalling through macromolecular protein complexes. Mol Immunol. doi: 10.1016/j.molimm.2014.06.033

Chen, L., Guo, S., Ranzer, M. J., & DiPietro, L. A. (2013). Toll-like receptor 4 has an essential role in early skin wound healing. J Invest Dermatol, 133(1), 258-267. doi: 10.1038/jid.2012.267

Choi, S. R., Chung, B. Y., Kim, S. W., Kim, C. D., Yun, W. J., Lee, M. W., . . . Chang, S. E. (2014). Activation of autophagic pathways is related to growth inhibition and senescence in cutaneous squamous cell carcinoma. Exp Dermatol, 23(10), 718-724. doi: 10.1111/exd.12515

Dotto, G. P. (2008). Notch tumor suppressor function. Oncogene, 27(38), 5115-5123. doi: 10.1038/onc.2008.225

Dotto, G. P. (2011). Calcineurin signaling as a negative determinant of keratinocyte cancer stem cell potential and carcinogenesis. Cancer Res, 71(6), 2029-2033. doi: 10.1158/0008-5472.CAN-10-3750

Dziunycz, P. J., Lefort, K., Wu, X., Freiberger, S. N., Neu, J., Djerbi, N., . . . Hofbauer, G. F. (2014). The oncogene ATF3 is potentiated by cyclosporine A and ultraviolet light A. J Invest Dermatol, 134(7), 1998-2004. doi: 10.1038/jid.2014.77

Ghosh, S., & Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. Cell, 109 Suppl, S81-96.

Ianaro, A., Tersigni, M., & D'Acquisto, F. (2009). New insight in LPS antagonist. Mini Rev Med Chem, 9(3), 306-317.

Ilmarinen, T., Hagstrom, J., Haglund, C., Auvinen, E., Leivo, I., Pitkaranta, A., & Aaltonen, L. M. (2014). Low Expression of Nuclear Toll-like Receptor 4 in Laryngeal Papillomas Transforming into Squamous Cell Carcinoma. Otolaryngol Head Neck Surg. doi: 10.1177/0194599814549730

Ivanov, V. N., Partridge, M. A., Huang, S. X., & Hei, T. K. (2011). Suppression of the proinflammatory response of metastatic melanoma cells increases TRAIL-induced apoptosis. J Cell Biochem, 112(2), 463-475. doi: 10.1002/jcb.22934

Jia, R. J., Cao, L., Zhang, L., Jing, W., Chen, R., Zhu, M. H., . . . Guo, Y. J. (2014). Enhanced myeloid differentiation factor 88 promotes tumor metastasis via induction of epithelial-mesenchymal transition in human hepatocellular carcinoma. Cell Death Dis, 5, e1103. doi: 10.1038/cddis.2014.71

Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., . . . Noble, P. W. (2005). Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat Med, 11(11), 1173-1179. doi: 10.1038/nm1315

Junttila, M. R., Li, S. P., & Westermarck, J. (2008). Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J, 22(4), 954-965. doi: 10.1096/fj.06-7859rev

Kageshita, T., Funasaka, Y., Ichihashi, M., Ishihara, T., Tokuo, H., & Ono, T. (2002). Differential expression of tissue factor and tissue factor pathway inhibitor in metastatic melanoma lesions. Pigment Cell Res, 15(3), 212-216.

Kalinin, A., Marekov, L. N., & Steinert, P. M. (2001). Assembly of the epidermal cornified cell envelope. J Cell Sci, 114(Pt 17), 3069-3070.

Kluwe, J., Mencin, A., & Schwabe, R. F. (2009). Toll-like receptors, wound healing, and carcinogenesis. J Mol Med (Berl), 87(2), 125-138. doi: 10.1007/s00109-008-0426-z

Kolar, M., Szabo, P., Dvorankova, B., Lacina, L., Gabius, H. J., Strnad, H., . . . Smetana, K., Jr. (2012). Upregulation of IL-6, IL-8 and CXCL-1 production in dermal fibroblasts by normal/malignant epithelial cells in vitro: Immunohistochemical and transcriptomic analyses. Biol Cell, 104(12), 738-751. doi: 10.1111/boc.201200018

Koster, M. I., & Roop, D. R. (2007). Mechanisms regulating epithelial stratification. Annu Rev Cell Dev Biol, 23, 93-113. doi: 10.1146/annurev.cellbio.23.090506.123357

Kuphal, S., Martyn, A. C., Pedley, J., Crowther, L. M., Bonazzi, V. F., Parsons, P. G., . . . Boyle, G. M. (2009). H-cadherin expression reduces invasion of malignant melanoma. Pigment Cell Melanoma Res, 22(3), 296-306. doi: 10.1111/j.1755-148X.2009.00568.x

Kypriotou, M., Huber, M., & Hohl, D. (2012). The human epidermal differentiation complex: cornified envelope precursors, S100 proteins and the 'fused genes' family. Exp Dermatol, 21(9), 643-649. doi: 10.1111/j.1600-0625.2012.01472.x

Lai, Y. H., He, R. Y., Chou, J. L., Chan, M. W., Li, Y. F., & Tai, C. K. (2014). Promoter hypermethylation and silencing of tissue factor pathway inhibitor-2 in oral squamous cell carcinoma. J Transl Med, 12, 237. doi: 10.1186/s12967-014-0237-7

Lambertini, C., Pantano, S., & Dotto, G. P. (2010). Differential control of Notch1 gene transcription by Klf4 and Sp3 transcription factors in normal versus cancer-derived keratinocytes. PLoS One, 5(4), e10369. doi: 10.1371/journal.pone.0010369

Lebre, M. C., van der Aar, A. M., van Baarsen, L., van Capel, T. M., Schuitemaker, J. H., Kapsenberg, M. L., & de Jong, E. C. (2007). Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. J Invest Dermatol, 127(2), 331-341. doi: 10.1038/sj.jid.5700530

Lee, E., Yi, J. Y., Chung, E., & Son, Y. (2010). Transforming growth factorbeta(1) transactivates EGFR via an H(2)O(2)-dependent mechanism in squamous carcinoma cell line. Cancer Lett, 290(1), 43-48. doi: 10.1016/j.canlet.2009.08.022

Lefort, K., & Dotto, G. P. (2004). Notch signaling in the integrated control of keratinocyte growth/differentiation and tumor suppression. Semin Cancer Biol, 14(5), 374-386. doi: 10.1016/j.semcancer.2004.04.017

Lim, X., & Nusse, R. (2013). Wnt signaling in skin development, homeostasis, and disease. Cold Spring Harb Perspect Biol, 5(2). doi: 10.1101/cshperspect.a008029

Loryman, C., & Mansbridge, J. (2008). Inhibition of keratinocyte migration by lipopolysaccharide. Wound Repair Regen, 16(1), 45-51. doi: 10.1111/j.1524-475X.2007.00290.x

Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. Cytokine, 42(2), 145-151. doi: 10.1016/j.cyto.2008.01.006

Macedo, L., Pinhal-Enfield, G., Alshits, V., Elson, G., Cronstein, B. N., & Leibovich, S. J. (2007). Wound healing is impaired in MyD88-deficient mice: a role for MyD88 in the regulation of wound healing by adenosine A2A receptors. Am J Pathol, 171(6), 1774-1788. doi: 10.2353/ajpath.2007.061048

Machida, H., Nakajima, S., Shikano, N., Nishio, J., Okada, S., Asayama, M., . . . Kubota, N. (2005). Heat shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin potentiates the radiation response of tumor cells grown as monolayer cultures and spheroids by inducing apoptosis. Cancer Sci, 96(12), 911-917. doi: 10.1111/j.1349-7006.2005.00125.x

Massi, D., & Panelos, J. (2012). Notch signaling and the developing skin epidermis. Adv Exp Med Biol, 727, 131-141. doi: 10.1007/978-1-4614-0899-4_10

Mittal, D., Saccheri, F., Venereau, E., Pusterla, T., Bianchi, M. E., & Rescigno, M. (2010). TLR4-mediated skin carcinogenesis is dependent on immune and radioresistant cells. EMBO J, 29(13), 2242-2252. doi: 10.1038/emboj.2010.94

Moriyama, M., Durham, A. D., Moriyama, H., Hasegawa, K., Nishikawa, S., Radtke, F., & Osawa, M. (2008). Multiple roles of Notch signaling in the regulation of epidermal development. Dev Cell, 14(4), 594-604. doi: 10.1016/j.devcel.2008.01.017

Muehleisen, B., Jiang, S. B., Gladsjo, J. A., Gerber, M., Hata, T., & Gallo, R. L. (2012). Distinct innate immune gene expression profiles in non-melanoma skin cancer of immunocompetent and immunosuppressed patients. PLoS One, 7(7), e40754. doi: 10.1371/journal.pone.0040754

Muto, J., Morioka, Y., Yamasaki, K., Kim, M., Garcia, A., Carlin, A. F., . . . Gallo, R. L. (2014). Hyaluronan digestion controls DC migration from the skin. J Clin Invest, 124(3), 1309-1319. doi: 10.1172/JCI67947

Nestle, F. O., Conrad, C., Tun-Kyi, A., Homey, B., Gombert, M., Boyman, O., . . . Gilliet, M. (2005). Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med, 202(1), 135-143. doi: 10.1084/jem.20050500

Nguyen, B. C., Lefort, K., Mandinova, A., Antonini, D., Devgan, V., Della Gatta, G., . . . Dotto, G. P. (2006). Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. Genes Dev, 20(8), 1028-1042. doi: 10.1101/gad.1406006

O'Neill, L. A., & Bowie, A. G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol, 7(5), 353-364. doi: 10.1038/nri2079

Okuyama, R., Tagami, H., & Aiba, S. (2008). Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. J Dermatol Sci, 49(3), 187-194. doi: 10.1016/j.jdermsci.2007.05.017

Panzer, R., Blobel, C., Folster-Holst, R., & Proksch, E. (2014). TLR2 and TLR4 expression in atopic dermatitis, contact dermatitis and psoriasis. Exp Dermatol, 23(5), 364-366. doi: 10.1111/exd.12383

Park, B. S., Song, D. H., Kim, H. M., Choi, B. S., Lee, H., & Lee, J. O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature, 458(7242), 1191-1195. doi: 10.1038/nature07830

Pasparakis, M., Haase, I., & Nestle, F. O. (2014). Mechanisms regulating skin immunity and inflammation. Nat Rev Immunol, 14(5), 289-301. doi: 10.1038/nri3646

Pivarcsi, A., & Homey, B. (2005). Chemokine networks in atopic dermatitis: traffic signals of disease. Curr Allergy Asthma Rep, 5(4), 284-290.

Prasad, N. B., Fischer, A. C., Chuang, A. Y., Wright, J. M., Yang, T., Tsai, H. L., . . . Tufaro, A. P. (2014). Differential expression of degradome components in cutaneous squamous cell carcinomas. Mod Pathol, 27(7), 945-957. doi: 10.1038/modpathol.2013.217

Restivo, G., Nguyen, B. C., Dziunycz, P., Ristorcelli, E., Ryan, R. J., Ozuysal, O. Y., . . . Dotto, G. P. (2011). IRF6 is a mediator of Notch pro-differentiation and tumour suppressive function in keratinocytes. EMBO J, 30(22), 4571-4585. doi: 10.1038/emboj.2011.325

Rheinwald, J. G., & Beckett, M. A. (1981). Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. Cancer Res, 41(5), 1657-1663.

Ruf, W. (2003). Tissue factor-dependent signaling in tumor biology. Pathophysiol Haemost Thromb, 33 Suppl 1, 28-30. doi: 73286

Ruf, W., Seftor, E. A., Petrovan, R. J., Weiss, R. M., Gruman, L. M., Margaryan, N. V., . . . Hendrix, M. J. (2003). Differential role of tissue factor pathway inhibitors 1 and 2 in melanoma vasculogenic mimicry. Cancer Res, 63(17), 5381-5389.

Suga, H., Sugaya, M., Fujita, H., Asano, Y., Tada, Y., Kadono, T., & Sato, S. (2014). TLR4, rather than TLR2, regulates wound healing through TGF-beta and CCL5 expression. J Dermatol Sci, 73(2), 117-124. doi: 10.1016/j.jdermsci.2013.10.009

Swann, J. B., Vesely, M. D., Silva, A., Sharkey, J., Akira, S., Schreiber, R. D., & Smyth, M. J. (2008). Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. Proc Natl Acad Sci U S A, 105(2), 652-656. doi: 10.1073/pnas.0708594105

Szczepanski, M. J., Czystowska, M., Szajnik, M., Harasymczuk, M., Boyiadzis, M., Kruk-Zagajewska, A., . . . Whiteside, T. L. (2009). Triggering of Toll-like receptor 4 expressed on human head and neck squamous cell carcinoma promotes tumor development and protects the tumor from immune attack. Cancer Res, 69(7), 3105-3113. doi: 10.1158/0008-5472.CAN-08-3838

Takazawa, Y., Kiniwa, Y., Ogawa, E., Uchiyama, A., Ashida, A., Uhara, H., . . . Okuyama, R. (2014). Toll-like receptor 4 signaling promotes the migration of human melanoma cells. Tohoku J Exp Med, 234(1), 57-65.

Takeuchi, T., Liang, S. B., Matsuyoshi, N., Zhou, S., Miyachi, Y., Sonobe, H., & Ohtsuki, Y. (2002). Loss of T-cadherin (CDH13, H-cadherin) expression in cutaneous squamous cell carcinoma. Lab Invest, 82(8), 1023-1029.

Takeuchi, T., Liang, S. B., & Ohtsuki, Y. (2002). Downregulation of expression of a novel cadherin molecule, T-cadherin, in basal cell carcinoma of the skin. Mol Carcinog, 35(4), 173-179. doi: 10.1002/mc.10088

Toll, A., Masferrer, E., Hernandez-Ruiz, M. E., Ferrandiz-Pulido, C., Yebenes, M., Jaka, A., . . . Hernandez-Munoz, I. (2013). Epithelial to mesenchymal transition markers are associated with an increased metastatic risk in primary cutaneous squamous cell carcinomas but are attenuated in lymph node metastases. J Dermatol Sci, 72(2), 93-102. doi: 10.1016/j.jdermsci.2013.07.001

Vabulas, R. M., Wagner, H., & Schild, H. (2002). Heat shock proteins as ligands of toll-like receptors. Curr Top Microbiol Immunol, 270, 169-184.

Weng, H., Deng, Y., Xie, Y., Liu, H., & Gong, F. (2013). Expression and significance of HMGB1, TLR4 and NF-kappaB p65 in human epidermal tumors. BMC Cancer, 13, 311. doi: 10.1186/1471-2407-13-311

Wu, X., Nguyen, B. C., Dziunycz, P., Chang, S., Brooks, Y., Lefort, K., . . . Dotto, G. P. (2010). Opposing roles for calcineurin and ATF3 in squamous skin cancer. Nature, 465(7296), 368-372. doi: 10.1038/nature08996

Yusuf, N., Nasti, T. H., Long, J. A., Naseemuddin, M., Lucas, A. P., Xu, H., & Elmets, C. A. (2008). Protective role of Toll-like receptor 4 during the initiation stage of cutaneous chemical carcinogenesis. Cancer Res, 68(2), 615-622. doi: 10.1158/0008-5472.CAN-07-5219

4. CD32B as negative regulator for keratinocyte proliferation

4.1. Research letter

Fcγ receptor II (CD32) regulates keratinocyte proliferation and differentiation

Freiberger, Sandra N.[1]; Iotzova-Weiss, Guergana[1]; Dziunycz, Piotr J.[1]; Hofbauer, Günther F.L.[1]*

[1] Department of Dermatology, University Hospital Zurich, Switzerland

* Corresponding author: Günther F. L. Hofbauer, Department of Dermatology, University Hospital Zurich, Gloriastrasse 31, 8091 Zurich, Switzerland. Phone: +41 44 2551111; Fax: +41 44 2559985 E-mail: Hofbauer@usz.ch

Short title: CD32's role in keratinocytes

Abbreviations:
SCC = Squamous cell carcinoma
FcγR = Fc gamma receptor

Fcγ receptors are known to recognize antigen/antibody complexes and trigger signaling cascades to activate or inhibit gene transcription resulting in several immune responses like the release of inflammatory mediators, the activation of phagocytosis or the activation of the complement cascade (Nimmerjahn and Ravetch, 2008). The collectivity of Fcγ receptors includes three different groups, CD64 (FcγRI), CD32 (FcγRII) and CD16 (FcγRIII), which show structural diversity. More specifically, FcγRII is encoded by three homologous genes in humans (FcγRIIA, FcγRIIB, FcγRIIC), where one of them has three alternative splice variants (FcγRIIBa, FcγRIIBb, FcγRIIBc). All Fcγ receptors are integral membrane glycoproteins. While FcγRI has a high affinity to its ligand, FcγRII and FcγRIII are low-affinity receptors (Ravetch and Kinet, 1991). In our study we will mainly focus on FcγRIIB (CD32B), which is an inhibitory receptor usually expressed on hematopoietic cells where it regulates B-cell-, T-cell- and mast cell activation (Daeron et al., 2008). A study in mast cells showed that FcγRIIB can inhibit cell proliferation when co-aggregated with c-kit, a receptor tyrosine kinase (Malbec et al., 1999).

CD32 is expressed on keratinocytes (Cauza et al., 2002) and is upregulated by treatment with the chemical agent sulfur mustard (Cowan et al., 1998) which in turn leads to keratinocyte differentiation (Popp et al., 2011). However, the role of CD32 isoforms and their signaling pathways in keratinocytes and in the context of cancer, especially cutaneous squamous cell carcinoma (SCC), are unknown. In this report, we present CD32 as a negative regulator of keratinocyte proliferation and show its involvement in keratinocyte differentiation. We incubated normal keratinocytes and the SCC13 cell line with either isotype control or a blocking antibody against CD32 for 24 hrs and analyzed proliferation by BrdU incorporation. Moreover, we knocked down CD32B with shRNA followed by assessing proliferation. Interestingly, keratinocyte proliferation was enhanced by blocking of CD32 with the specific blocking antibody as well as by CD32B knock-down, while proliferation of SCC13 was not affected by either blocking CD32 or knock-down of CD32B (Fig. 1A). Assuming either lack of expression or function of CD32 in SCC13 cells, we analyzed protein expression of CD32A, CD32B and CD32C on keratinocytes, the keratinocyte cell line HaCaT and the SCC13 cell line. As we found all CD32 isoforms higher expressed in SCC13 cells than in keratinocytes (Fig. 1B), we assume that CD32 on SCC13 either does not signal or cannot further increase the high proliferation rate of this cancer cell line in a measurable degree. Next we overexpressed CD32 isoforms in keratinocytes and two SCC cell lines to analyze their proliferation

behavior. CD32 overexpression in keratinocytes as well as in two SCC cell lines decreased cellular proliferation (Fig. 1C).

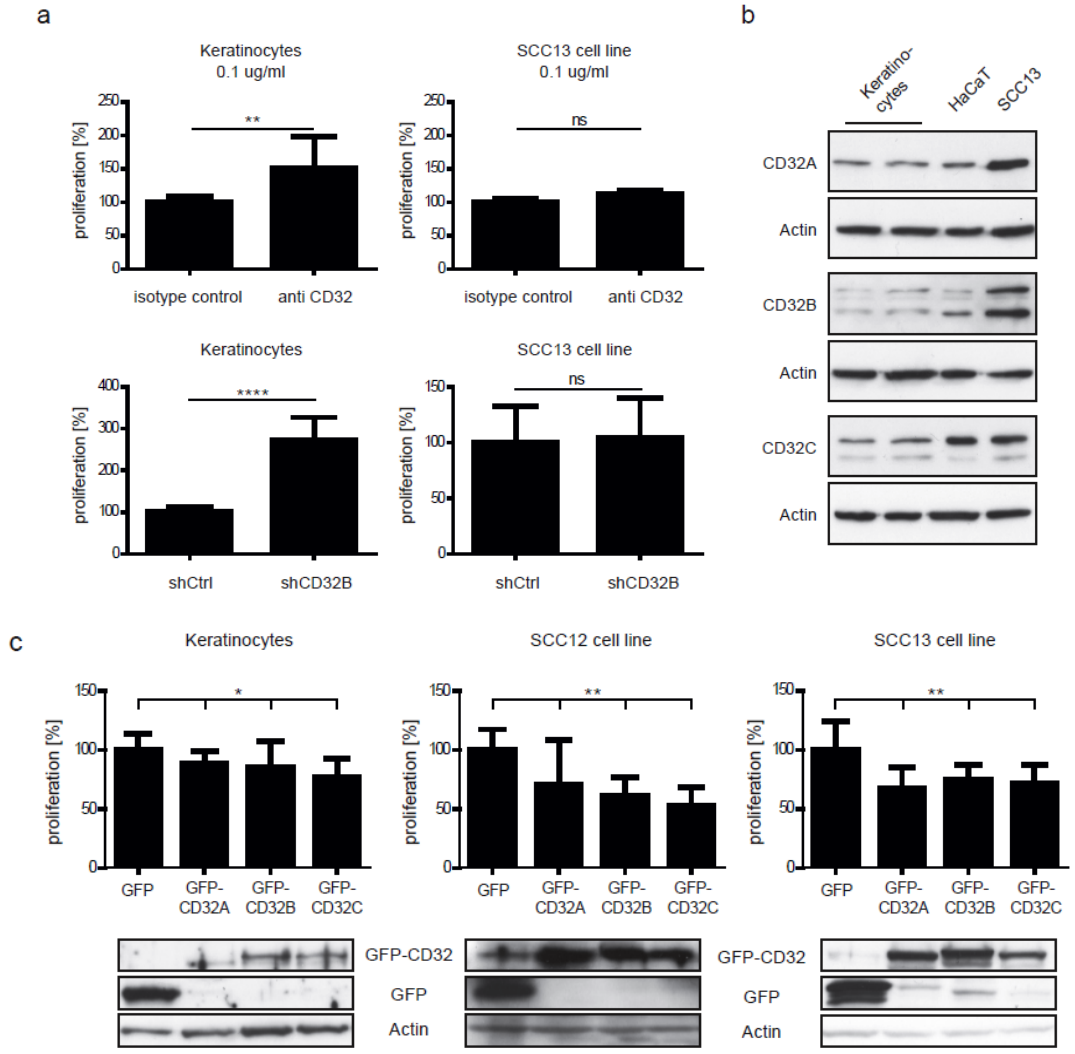


Fig.1: CD32 regulates proliferation of keratinocytes. a) Keratinocytes and SCC13 cells were incubated with 0.1 ug/ml blocking antibody against CD32 or corresponding isotype control for 24 hrs (upper panel). CD32B was knocked down in keratinocytes and SCC13 cells with shRNA (lower panel). Proliferation was assessed by BrdU incorporation. Graphs represent mean +/- SD of three independent experiments. Data were analyzed by student´s t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. b) Total protein extracts of keratinocytes, HaCaT and SCC13 cells were analyzed for expression of CD32 isoforms. Actin was used as loading control. c) CD32 isoforms were overexpressed in keratinocytes, SCC12 and SCC13 cells. Western blots confirm overexpression of CD32 isoforms. Proliferation was assessed by BrdU incorporation. Graphs represent mean +/- SD of three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnet's multiple-comparison test. * = $p<0.05$, ** = $p<0.01$.

Terminal keratinocytes stop growing and start differentiating. To investigate whether CD32 expression is associated with the differentiation status, we analyzed CD32 protein expression on growing and differentiated keratinocytes. Here, we found CD32 isoforms to be higher expressed when driving keratinocytes towards differentiation (Fig. 2A). CD32B immunoreactivity also increased with epidermal differentiation on normal skin with highest expression in upper layers of the epidermis (Fig. 2B). This correlation with differentiation corresponds with the functional cell studies above where CD32 inversely correlates with proliferation (Fig 1A, 1C).

To translate our cellular data into the clinical setting, we investigated CD32B expression in normal human skin and human SCC using a tissue microarray consisting of over 200 evaluable SCC samples in different differentiation states. We found CD32B to be higher expressed in normal skin compared to SCC as measured by highest immunoreactivity in each biopsy (Fig. 2C). This reduced expression of CD32B in human SCC corresponds to the inverse role for CD32B we had observed in our functional cell studies.

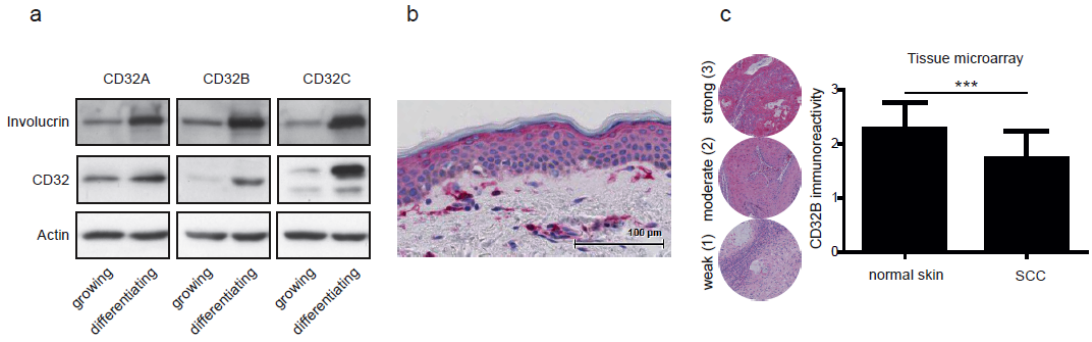


Fig.2: CD32 expression is associated with keratinocyte differentiation. a) Expression of CD32 isoforms was analyzed in total protein extracts from growing and differentiating keratinocytes. Involucrin was used as differentiation marker; actin was used as loading control. b) Normal human skin was stained for CD32B by IHC. c) Tissue microarray was stained for CD32B and analyzed semi-quantitatively. The graph shows mean +/- SD. Data were analyzed by $\chi 2$-test. *** = $p<0.001$.

In summary, CD32B shows differential expression in normal human skin and human SCC. Functional data shows an inverse regulatory role for CD32B in keratinocyte proliferation. Differentiation in turn correlates with CD32 expression. While a previous study found CD32 to elicit growth arrest and differentiation in myeloblastic leukemia cells (Wightman et al., 2002), we believe to report a novel role for CD32B, a well-known immunoglobulin receptor, in keratinocytes and SCC.

4.2. References

Cauza, K., Grassauer, A., Hinterhuber, G., Horvat, R., Rappersberger, K., Wolff, K., and Foedinger, D. (2002). FcgammaRIII expression on cultured human keratinocytes and upregulation by interferon-gamma. The Journal of investigative dermatology 119, 1074-1079.

Cowan, F.M., Broomfield, C.A., and Smith, W.J. (1998). Sulfur mustard exposure enhances Fc receptor expression on human epidermal keratinocytes in cell culture: implications for toxicity and medical countermeasures. Cell biology and toxicology 14, 261-266.

Daeron, M., Jaeger, S., Du Pasquier, L., and Vivier, E. (2008). Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev 224, 11-43.

Malbec, O., Fridman, W.H., and Daeron, M. (1999). Negative regulation of c-kit-mediated cell proliferation by Fc gamma RIIB. J Immunol 162, 4424-4429.

Nimmerjahn, F., and Ravetch, J.V. (2008). Fcgamma receptors as regulators of immune responses. Nat Rev Immunol 8, 34-47.

Popp, T., Egea, V., Kehe, K., Steinritz, D., Schmidt, A., Jochum, M., and Ries, C. (2011). Sulfur mustard induces differentiation in human primary keratinocytes: opposite roles of p38 and ERK1/2 MAPK. Toxicology letters 204, 43-51.

Ravetch, J.V., and Kinet, J.P. (1991). Fc receptors. Annu Rev Immunol 9, 457-492.

Wightman, J., Roberson, M.S., Lamkin, T.J., Varvayanis, S., and Yen, A. (2002). Retinoic acid-induced growth arrest and differentiation: retinoic acid up-regulates CD32 (Fc gammaRII) expression, the ectopic expression of which retards the cell cycle. Mol Cancer Ther 1, 493-506.

5. The oncogen ATF3 is potentiated by cyclosporine A and ultraviolet light A

The immunosuppressive calcineurin inhibitor cyclosporine A (CsA) is known to promote SCC development in OTR. Furthermore, the transcription factor ATF3 is highly expressed in SCCs and is negatively controlled by calcineurin signaling. Like SCC in immunocompetent people, SCC in OTR arises mainly on sun-exposed surfaces of the skin. In this project we investigated the effect of CsA and UVA light on the transcription factor ATF3 and propose a model on how the combination of both of them potentiates ATF3 expression and mediates SCC development by different pathways.

Contribution to the manuscript:
I contributed to this project by isolating primary cells from tissue, by irradiating organ cultures and by performing immunofluorescence staining and microscopy.

This study was published in the Journal of Investigative Dermatology (JID).

ORIGINAL ARTICLE

The Oncogene ATF3 Is Potentiated by Cyclosporine A and Ultraviolet Light A

Piotr J. Dziunycz[1], Karine Lefort[2], Xunwei Wu[3], Sandra N. Freiberger[1], Johannes Neu[1], Nadia Djerbi[1], Guergana Iotzowa-Weiss[1], Lars E. French[1], Gian-Paolo Dotto[2,3] and Günther F.L. Hofbauer[1]

Cutaneous squamous cell carcinoma (SCC) represents the most important cutaneous complication following organ transplantation. It develops mostly on sun-exposed areas. A recent study showed the role of activating transcription factor 3 (ATF3) in SCC development following treatment with calcineurin inhibitors. It has been reported that ATF3, which may act as an oncogene, is under negative calcineurin/nuclear factor of activated T cells (NFAT) control and is upregulated by calcineurin inhibitors. Still, these findings do not fully explain the preferential appearance of SCC on chronically sun-damaged skin. We analyzed the influence of UV radiation on ATF3 expression and its potential role in SCC development. We found that ATF3 is a specifically induced AP1 member in SCC of transplanted patients. Its expression was strongly potentiated by combination of cyclosporine A and UVA treatment. UVA induced ATF3 expression through reactive oxygen species-mediated nuclear factor erythroid 2–related factor 2 (NRF2) activation independently of calcineurin/NFAT inhibition. Activated NRF2 directly binds to ATF3 promoter, thus inducing its expression. These results demonstrate two mechanisms that independently induce and, when combined together, potentiate the expression of ATF3, which may then force SCC development. Taking into account the previously defined role of ATF3 in the SCC development, these findings may provide an explanation and a mechanism for the frequently observed burden on SCCs on sun-exposed areas of the skin in organ transplant recipients treated by calcineurin inhibitors.

Journal of Investigative Dermatology (2014) **134**, 1998–2004; doi:10.1038/jid.2014.77; published online 13 March 2014

INTRODUCTION

Squamous cell carcinoma (SCC) of the skin is one of the most common cancers at large and the second most common cancer of the skin. Predilection sites for SCC are the sun-exposed areas of the skin, such as the face, the balding head, the dorsum of the hands, and the lower arms. This clinical distribution highlights chronic low-level UV light exposure as a major factor for SCC formation.

Following organ transplantation, SCC emerges as the most common cancer: SCC occurs 65- to 250-fold more frequently in organ transplant recipients (OTRs) than in the general population (Kempf *et al.*, 2012). As a result, 20–75% of OTRs are affected by at least one SCC within 20 years of transplantation (Hofbauer *et al.*, 2010). Immunosuppressive drugs drive this greatly increased formation of SCC in OTR. Azathioprine photosensitizes cells to UVA and shows clinically relevant effects at the molecular level on keratinocytes (Hofbauer *et al.*, 2012). Calcineurin inhibition is the cornerstone of most immunosuppressive regimes. A number of studies have shown that deregulation of calcineurin-NFAT signaling has an important role in tumorigenesis (Dotto, 2011). The most commonly used inhibitor of calcineurin, cyclosporine A (CsA), directly induces tumor growth in murine models (Wu *et al.*, 2010) and increases the secretion of transforming growth factor-β and vascular endothelial growth factor conducive to SCC formation (Hofbauer, 2010). Although cyclosporine promotes SCC formation, a switch to mammalian target of rapamycin inhibitors such as rapamycin reduces SCC formation in OTR (Euvrard *et al.*, 2012). Recently, calcineurin inhibition was recognized to selectively induce the expression of activating transcription factor 3 (ATF3)—a member of the 'enlarged' AP-1 family (Wu *et al.*, 2010). ATF3 downregulates p53 expression by direct negative regulation of p53 messenger RNA (mRNA) expression and thus increases SCC formation *in vitro* in a mouse model and in human SCC (Wu *et al.*, 2010).

Although immunosuppressive drugs have a major role in SCC formation in OTR, these patients still show a predilection for skin areas with chronic sun damage (Lindelof *et al.*, 2005). We therefore speculated that a synergistic effect between UV light and immunosuppression causes the predilection of SCC in OTR for chronically sun-exposed skin.

[1]Department of Dermatology, University Hospital Zurich, Zurich, Switzerland; [2]Department of Biochemistry, University of Lausanne, Epalinges, Switzerland and [3]Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard University, Charlestown, Massachusetts, USA

Correspondence: Piotr Dziunycz, Department of Dermatology, University Hospital Zurich, Gloriastrasse 31, 8091 Zurich, Switzerland. E-mail: piotr.dziunycz@usz.ch

Abbreviations: ATF3, activating transcription factor 3; CsA, cyclosporine A; NRF2, nuclear factor erythroid 2–related factor 2; OTR, organ transplant recipient; ROS, reactive oxygen species; SCC, squamous cell carcinoma

Received 11 October 2013; revised 23 December 2013; accepted 21 January 2014; accepted article preview online 7 February 2014; published online 13 March 2014

Here we found that UVA induces ATF3 expression by induction of nuclear factor erythroid 2–related factor 2 (NRF2) binding to the ATF3 promoter. The Nrf2-Keap1 (Kelch-like erythroid cell–derived protein with CNC homology (ECH)–associated protein 1) signaling pathway is one of the most important cell defense and survival pathways. Nrf2 can protect cells and tissues from a variety of toxicants and carcinogens by increasing the expression of a number of cytoprotective genes (Jaramillo and Zhang, 2013). Just as Nrf2 protects normal cells, studies have shown that Nrf2 may also protect cancer cells from chemotherapeutic agents and facilitate cancer progression. Nrf2 is aberrantly accumulated in many types of cancer, and its expression is associated with a poor prognosis in patients (Jaramillo and Zhang, 2013).

Our experiments reveal that UVA and cyclosporin potentiate expression of the transcription factor ATF3, suggesting an explanation for the clinically observed increase of SCC on the sun-damaged skin of OTRs.

RESULTS

ATF3 is a selectively upregulated AP1 family member in SCC of immunosuppressed patients

The real-time PCR analysis performed on SCCs from immunocompetent and immunosuppressed patients showed significant and specific upregulation of ATF3 expression in the OTR group (Figure 1a). The samples were analyzed for the expression of several members of the 'enlarged' AP-1 family; however, there was no increase in mRNA expression for ATF2, ATF4, c-Jun, or c-Fos. As ATF3 is known to function as a transcription factor (Thompson *et al.*, 2009), an open question was whether the observed increase in ATF3 mRNA level affects the expression of downstream targets of ATF3. Several putative ATF3 downstream genes have been identified, among them GADD153 (Wolfgang *et al.*, 1997). Interestingly, GADD153 mRNA was also increased in the OTR group in parallel to the increase of ATF3 mRNA expression (Figure 1b).

We then aimed to identify putative ATF3-regulated genes in normal human keratinocytes. A complementary DNA microarray analysis of keratinocytes overexpressing ATF3 and keratinocytes with downregulated expression of ATF3 suggested four putative ATF3 downstream targets (see Supplementary Table S1 online). Among them, Vanin-1 and potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 (KCNN4) were upregulated in the ATF3-overexpressing cells and downregulated in the siATF3-treated cells (Supplementary Table S1 online). Real-time PCR expression analysis of Vanin-1 and KCNN4 showed a clear upregulation of mRNA expression for these two genes in OTRs (Figure 1c) in line with the increased ATF3 mRNA expression observed in the same samples.

ATF3 expression is potentiated by CsA and UVA treatment

Consistent with previously published findings (Wu *et al.*, 2010), CsA-treated cells showed increased ATF3 mRNA and protein levels in a dose-dependent manner (Figure 2a and b). In the study by Wu *et al.*, it was also demonstrated that such CsA-driven induction of ATF3 has a major influence on SCC development. As UV radiation is one of the most important risk factors for SCC development, we exposed the primary human keratinocytes to low doses of UVA and UVB irradiation. Interestingly, UVA treatment significantly elevated ATF3 mRNA and protein expression (Figure 2c and d), whereas UVB had only moderate effects on ATF3 mRNA levels (Supplementary Figure S1 online). When low levels of CsA and UVA treatment of cultured primary human keratinocytes were combined, the upregulation of ATF3 expression was potentiated: the UVA- and CsA-treated cells expressed ATF3 at higher mRNA and protein levels than did keratinocytes treated with UVA or CsA alone (Figure 2e and f). These findings were next verified in a model of organ culture of normal human skin (Figure 2g and h). CsA treatment induced ATF3 expression in the epidermis. When normal skin was irradiated with low doses of UVA, only a slight induction of ATF3 was observed at the protein level. However, when skin was pretreated with CsA and then exposed to UVA irradiation, the combined treatment strongly induced ATF3 expression at both the mRNA and protein level in the epidermis. This synergistic impact of UVA and CsA on gene expression was very potent and specific for ATF3 alone. Expression of other AP-1 family members was not influenced by the combined UVA and CsA treatment (see Supplementary Data online).

UVA-mediated induction of ATF3 expression requires reactive oxygen species (ROS) formation and NRF2 activation

UVA influences the cellular processes mostly by the induction of stress, and particularly by the formation of ROS. Primary human keratinocytes exposed to UVA radiation strongly induce the formation of ROS. This, however, can be decreased by pretreatment of the cells with β-carotene and to a lesser extent with α-tocopherol, both of which serve as ROS scavengers (Figure 3a). Interestingly, once cells were pretreated with either of these two scavengers and then exposed to UVA irradiation, UV light was less potent in its induction of ATF3 expression (Figure 3b). The inhibition of ATF3 expression correlated with the strength of the compound as a ROS scavenger. β-Carotene, which is more potent in the reduction of ROS formation (Figure 3a), also strongly inhibited ATF3 formation after UVA irradiation (Figure 3b). This effect was less pronounced in the case of pretreatment with α-tocopherol, which is also reported to be a less potent ROS scavenger than β-carotene.

These results were verified in skin culture. Epidermis of the normal skin exposed to UVA at 50 J cm^{-2} increased its expression of ATF3. However, ATF3 induction was strongly reduced by pretreatment with β-carotene (Figure 3c). This effect was also confirmed at the protein level (Figure 3d). The cell injury caused by UVA irradiation is known to induce the expression of genes encoding antioxidant and/or phase 2 detoxifying enzymes (Gruber *et al.*, 2010). The main transcription factor involved in this response is nuclear factor erythroid 2–related factor 2 (Nrf2; Marrot *et al.*, 2008). In our study, UVA irradiation induced the protein expression of NRF2 in the epidermis, and this effect could be counteracted by an antioxidative treatment with β-carotene (Figure 3d).

Previous reports suggested a close relation between NRF2 and ATF3 expression in other cells such as inflammatory cells

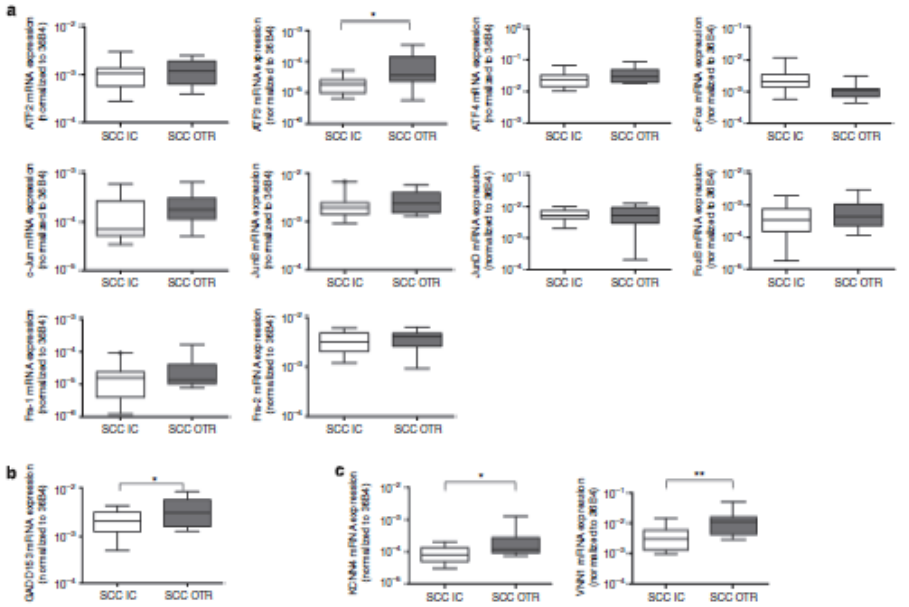


Figure 1. Activating transcription factor 3 (ATF3) is a specifically increased AP1 member in squamous cell carcinoma (SCC) of organ transplant recipients (OTRs). (a) Messenger RNA (mRNA) levels of ATF3 were measured in the epidermis of SCC derived from immunocompetent (IC) and OTR patients (SCC IC and SCC OTR, respectively). Out of the 'enlarged' AP1 family members: the levels of ATF3, ATF2, ATF4, c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2 were measured. Of the 10 tested genes, only ATF3 showed significant differences in expression between the two groups of patients. (b) The samples were then used for the measurement of putative ATF3 downstream target expression. GADD45 β was upregulated in the SCCs of OTRs. (c) Selection of other putative ATF3 downstream targets was based on complementary DNA microarray analysis after downregulation or overexpression of ATF3 in primary human keratinocytes (Supplementary Table S1 online). The analysis showed a significant increase in Vanin-1 (VNN1) and potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 (KCNN4) mRNA in cyclosporine-treated patients (SCC OTR) as compared with the IC group of patients (SCC IC). This induction paralleled the increased levels of ATF3 in the OTR group. *$P<0.05$; **$P<0.01$.

(Hoetzenecker *et al.*, 2012). To verify whether UV induces ATF3 expression through NRF2 activation, primary human keratinocytes were treated with NRF2-specific small interfering RNA (siRNA). Cells treated with control siRNA and exposed to UVA induced the expression of both NRF2 and ATF3. However, the UVA-mediated ATF3 induction was inhibited in NRF2 knockdown keratinocytes (Figure 3e). To verify these results, cells were treated with tert-butylhydroquinone, which is a known NRF2 stabilizer (Khodagholi and Tusi, 2011). The NRF2 stabilization by tert-butylhydroquinone increased the expression of ATF3. This, however, was abolished in NRF2 knockdown cells (Figure 3f).

ATF3 is a direct downstream target of NRF2 in keratinocytes upon UVA irradiation

Sequence analysis of the proximal region of the human Atf3 gene promoter revealed the presence of 'canonical' antioxidant response element (ARE)-binding sites located at −3.5 and −2.9 kb from the transcription start site (Figure 4a). To verify whether NRF2 binds to these sites, we performed chromatin immunoprecipitation assays with extracts from human primary keratinocytes that were either untreated or irradiated with UVA (Figure 4b). The analysis showed binding of the NRF2 protein to both of the predicted motifs within the promoter (−3.5 and −2.9 kb position) upon UVA irradiation.

CsA and UVA induce ATF3 independently from each other

CsA treatment of primary human keratinocytes slightly induced ROS formation as assessed by the ROS assay (Figure 3g). However, this discrete ROS induction had no impact on NRF2 protein expression. In conclusion, CsA treatment did not induce NRF2 protein expression (Figure 3h). On the other hand, UVA irradiation of primary human keratinocytes had no effect on NFATc1 translocation (Supplementary Figure S3 online). In untreated keratinocytes, NFATc1 is located in the cytoplasm. Upon activation of calcineurin, e.g., by addition of

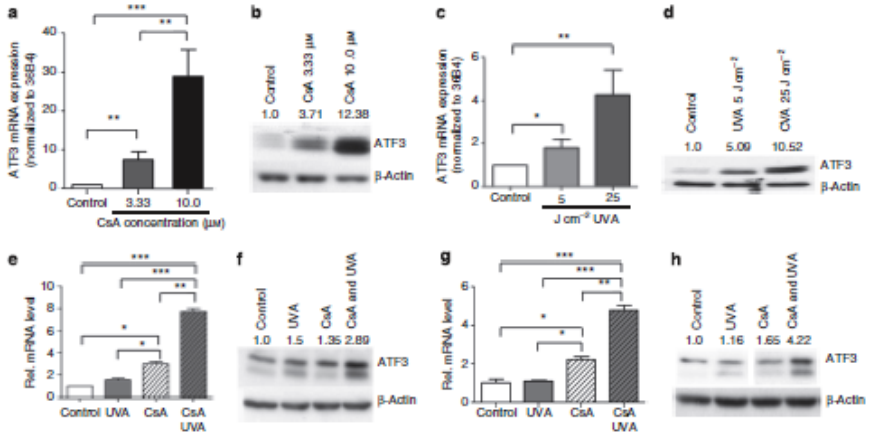


Figure 2. Activating transcription factor 3 (ATF3) expression is potentiated by cyclosporine A (CsA) and UVA treatment. Primary human keratinocytes were treated with increasing CsA concentrations for 6 hours. After this time period, the cells were homogenized and the expression of ATF3 was measured at the (a) messenger RNA (mRNA) and (b) protein level. Primary human keratinocytes were exposed to UVA radiation at 5 and 25 J cm^{-2}. Six hours after irradiation, cells were homogenized and ATF3 expression was measured at the (c) mRNA and (d) protein level. The results showed a significant dose-dependent increase in ATF3 expression after UVA irradiation. (e) Combined low-level CsA and UVA treatment potentiated ATF3 expression in HKCs at the mRNA and (f) protein level. The influence of combined CsA and UVA treatment was also measured in fresh-skin organ cultures. The abdominal skin obtained from healthy donors was incubated in semisolid culture. The skin was pretreated with CsA for 12 hours and then irradiated with UVA at the dose of 10 J cm^{-2}. Six hours following irradiation, the epidermis was separated from the underlying dermis by a brief heat treatment (Kolev *et al.*, 2008). The epidermis was then homogenized and the expression of ATF3 was measured at both the (g) mRNA and (h) protein level (the ATF3 lines were not originally run next to each other and have been juxtaposed in the figure). The combined low-level CsA and UVA treatment significantly increased ATF3 expression in the epidermis. *P<0.05; **P<0.01; ***P< 0.001. Rel., relative.

$Ca2^+$, NFATc1 translocates into the nucleus. This translocation is inhibited by pretreatment with calcineurin inhibitors such as CsA. Irradiation of primary human keratinocytes did not induce the nuclear translocation of NFATc1. On the other hand, when the cells were first treated with $Ca2^+$, thus activating NFATc1, UVA irradiation did not inhibit the translocation of NFATc1, suggesting a lack of UVA effect on calcineurin activity (Supplementary Figure S3 online).

DISCUSSION

It is well known that immunosuppressive treatment overall and particularly treatment with calcineurin inhibitors markedly increases the development of SCC (Dantal *et al.*, 1998). It is, however, noteworthy that, although the CsA treatment is systemic, the skin is the organ most affected by SCC (Morath *et al.*, 2004). On the skin, the predilection sites of SCC are skewed to the sun-exposed areas (Lindelof *et al.*, 2005). This indicates that solar irradiation is one of the critical factors driving SCC development.

ATF3 is a transcription factor with distinct, either oncogenic or antioncogenic, functions (Thompson *et al.*, 2009). In our previous study, ATF3 was identified as a CsA-induced oncogene that stimulates SCC development (Wu *et al.*, 2010). Our results demonstrate significantly increased ATF3 mRNA levels in the group of SCCs that were derived from OTRs. Interestingly, ATF3 was the only member of the 'enlarged' AP-1 family that was induced upon CsA treatment in clinical samples. Further analysis suggested a biological relevance of the increase in ATF3 expression. As this is a transcription factor, it may influence expression of several other genes, thus having an impact on the cell phenotype and the process of carcinogenesis. The analysis of our clinical samples suggests a biological relevance for this recently described role of ATF3 with isolated upregulation of ATF3 and of its putative downstream targets such as GADD153, Vanin-1, and KCNN1.

The previously described mechanism of activation of ATF3 involved NFATc1 inhibition. This proposed mechanism provides an explanation for the general increase of SCC development in OTRs; however, it does not explain the particular increase of SCC in the sun-exposed areas of skin in OTRs. UV radiation was recently recognized as a carcinogen along with X-rays and other hitherto recognized carcinogens (IARC, 2012). UVB is known more for its direct effect on DNA (de Gruijl, 2002). UVA recently gained more attention for its indirect effects by oxidation and is of importance mainly because of its relative abundance in ambient sun light. Recent work on azathioprine as well as other photosensitizing drugs (Hofbauer *et al.*, 2012) shows the relevance of drug-induced photosensitization to UVA in SCC formation in the long run. Here we show that low-level UVA radiation was similarly

potent in inducing ATF3 as compared with CsA treatment. Interestingly, when the CsA treatment was combined with UVA irradiation, ATF3 expression was markedly potentiated. UVA exposure induces ATF3 expression independently of the calcineurin–NFATc1 pathway. The UVA radiation levels that were potent in the induction of ATF3 expression did not inhibit, nor did they activate, the nuclear translocation of NFATc1. It is known, however, that UVA strongly induces formation of ROS (de Gruijl, 2002). We found that the UVA-mediated induction of ATF3 expression depends on ROS formation, and could be quenched by α-tocopherol and β-carotene. As UVB does not induce ROS formation to such an extent as UVA, our findings may also provide an explanation for only slight induction of ATF3 mRNA observed and for the lack of ATF3 protein expression after UVB irradiation. ATF3 gene expression has been shown to be under direct control of NRF2 in other cells such as monocytes or astrocytes (Hoetzenecker *et al.*, 2012). We found that NRF2 served as a link between UVA-mediated induction of ROS formation and ATF3 expression. Upon UVA irradiation and ROS formation, NRF2 protein is activated in the cultured primary keratinocytes as well as in the epidermis of normal skin, which leads to binding of NRF2 to the two predicted antioxidant response element motifs in the ATF3 gene promoter and induces ATF3 expression.

Besides the previously described calcineurin–NFATc1 inhibition–mediated induction of ATF3 expression, we demonstrate here an independent mechanism of ATF3 expression, which may be of biological relevance in the process of cutaneous carcinogenesis. It is important to mention that these two pathways act independently from each other. Although CsA treatment slightly induced oxidative stress in the cell, this effect was too low to stabilize NRF2; however, the UVA radiation doses that induce ATF3 expression do not interfere

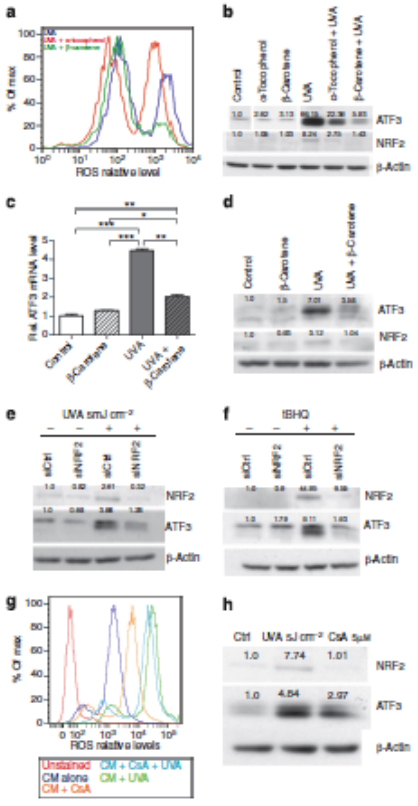


Figure 3. UVA induces activating transcription factor 3 (ATF3) expression through reactive oxygen species (ROS)-mediated activation of nuclear factor erythroid 2–related factor 2 (NRF2). (**a**) α-Tocopherol and β-carotene are potent ROS scavengers. HKCs were pretreated with α-tocopherol or β-carotene for 1 hour before UVA exposure. The cells were then irradiated with low-dose UVA (1 J cm^{-2}) followed by ROS measurement assay. β-Carotene acts as a more potent scavenger of ROS as compared with α-tocopherol. (**b**) Pretreatment for 1 hour of cultured human keratinocyte with α-tocopherol or β-carotene reduces UVA-mediated induction of ATF3 expression. After the pretreatment, cells were irradiated and left in the culture for another 6 hours, and then harvested (the ATF3 lines were not originally run next to each other and have been juxtaposed in the figure). (**c**) UVA-mediated induction of ATF3 expression in normal human skin was reduced by β-carotene treatment. Normal human skin was pretreated with β-carotene 1 hour before exposure to UVA. Six hours after irradiation, the epidermis was peeled from the dermis and homogenized for ATF3 expression. Real-time PCR showed significantly reduced induction of UVA-mediated ATF3 expression in β-carotene pretreated skin. (**d**) Normal skin pretreated with β-carotene and exposed to UVA showed significantly decreased protein levels of ATF3. UVA irradiation increased the protein level of NRF2, which was reduced by pretreatment with β-carotene. (**e**) HKCs were treated with NRF2-specific small interfering RNA (siRNA) (siNRF2) and then exposed to 5 J cm^{-2} of UVA. The control cells showed increased protein levels of NRF2 and ATF3. siRNA-mediated inhibition of NRF2 reduced the UVA-mediated induction of ATF3 expression. (**f**) Cultured cells were pretreated with NRF2-specific or control siRNA (siNRF2 and siCtrl, respectively) 6 hours before treatment with the known NRF2 stabilizer—tBHQ. Six hours after the tBHQ treatment, cells were homogenized for western blot. The analysis showed induction of ATF3 by tBHQ treatment following the induction of NRF2. siRNA-mediated inhibition of NRF2 expression inhibited the tBHQ-mediated increase in ATF3 expression. (**g**) The cyclosporine A (CsA)- and UVA-mediated pathways of ATF3 induction act independently of each other. Primary human keratinocytes were incubated in the presence of 5 μM CsA for 4 hours and then irradiated with low levels of UVA 5 J cm^{-2}. The ROS formation assay was performed directly after the irradiation, and the dye CM (CM-H2DCFDA) was used to assess the ROS levels. The UVA-irradiated cells (green curve) showed high levels of ROS, and pretreatment with CsA had no influence on ROS levels (blue curve). CsA treatment of primary keratinocytes induced low levels of ROS formation in the cells (orange curve). (**h**) Cultured HKCs were treated with either 5 μM CsA or UVA at 5 J cm^{-2}. Six hours after treatment, cells were homogenized, and ATF3 and NRF2 levels were measured by western blot. UVA irradiation stabilized NRF2 protein levels and induced ATF3 formation. CsA treatment had no influence on NRF2 protein levels; however, it induced ATF3 expression. *P<0.05; **P<0.01; ***P< 0.001. tBHQ, tert-butylhydroquinone.

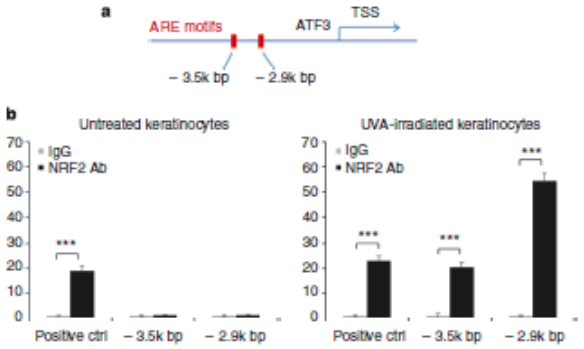


Figure 4. UVA irradiation induced binding of endogenous nuclear factor erythroid 2–related factor 2 (NRF2) to the activating transcription factor 3 (ATF3) locus within specific regions of chromatin organization. (**a**) Schematic illustration of chromatin immunoprecipitation (ChIP) results: TSS, transcription starting site; ARE-binding motifs are represented by the red bars. (**b**) ChIP assay was performed on untreated (left panel) and on UVA-irradiated human keratinocytes. The cell extracts were processed for ChIP assays using an antibody specific for NRF2, utilizing nonimmune IgGs as control (ctrl). PCR amplification of the two regions of the human ATF3 promoter encompassed the following ARE-binding sites: – 3.5 k bp: 5′-TGACACAGC-3′; – 2.9k bp: 5′-TGAGTGAGA-3′. The results are representative of two independent experiments. The relative amount of precipitated DNA, expressed in arbitrary units, was calculated after normalization for total input chromatin, according to the following formula: % total = $2^{\text{deltaCt}} \times 5$, where deltaCt = Ct (input) – Ct (immunoprecipitation). Ct, cycle threshold. *** $P<0.001$. ARE, antioxidant response element.

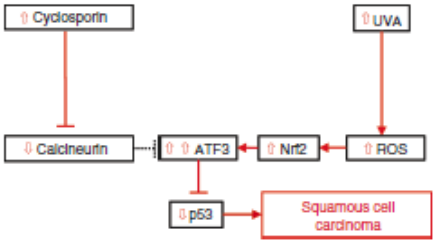


Figure 5. Schema representing the proposed mechanism of synergistic UVA- and cyclosporine A-mediated activating transcription factor 3 (ATF3) induction. Cyclosporine treatment through inhibition of calcineurin–NFAT signaling enhances expression of ATF3, a negative regulator of p53 gene transcription. The resulting downmodulation of p35 levels inhibits cancer cell senescence and can explain, in part, increased predisposition to cancer development. On the other hand, UVA radiation increases the reactive oxygen species (ROS) formation in the sun-exposed keratinocytes. This ROS formation leads to the activation of nuclear factor erythroid 2–related factor 2 (NRF2), which is a transcription factor that binds to ATF3 promoter and induces its expression. Thus, sun-exposed skin of cyclosporine-treated patients is particularly predisposed to skin cancer development. The proposed mechanism may explain this often observed clinical phenomena among organ transplant recipients.

with the NFATc1 activity. A schema representing our proposed mechanism of ATF3 induction by combined CsA treatment and UVA irradiation is summarized in Figure 5. In summary, our data suggest a potentiation of ATF3 by CsA and UVA as a mechanism for the greatly increased incidence of SCC in OTRs with a predilection for sun-damaged skin.

Here we show the importance of UVA irradiation in the skin carcinogenesis of CsA-treated patients. This study suggests a greater influence of UVA than of UVB in this process. The verification of this hypothesis in an animal model by comparing UVA with UVB influence in CsA-treated mice on skin cancer development remains to be done.

MATERIALS AND METHODS

Skin SCC samples

The cutaneous SCC (immunocompetent: $n = 19$, OTR: $n = 12$) samples were obtained at the Department of Dermatology of the University Hospital in Zurich, Switzerland, from clinical biopsies. All OTR patients were on CsA treatment for at least 5 years at the time of excision. Parts not needed for histological diagnosis were further processed. Normal skin was obtained from healthy donors undergoing abdominal reconstructive surgery. Institutional board approval as well as written informed patient consent were obtained for all the analyses.

Cell and organ cultures, plasmids, viruses, and siRNA

Conditions for generation and culturing of primary human keratinocytes were reported previously (Dziunycz *et al.*, 2010). Infection with retroviruses expressing ATF3 together with corresponding controls was described previously (Wu *et al.*, 2010). CsA (Novartis), α-tocopherol, and tert-butylhydroquinone (Sigma, Bucks, Switzerland) were dissolved in ethanol and stored in a stock solution at −20 °C. β-Carotene (Sigma) was always freshly dissolved in chloroform before the experiments. For knockdown experiments, cells were transfected as described (Restivo *et al.*, 2011) with siRNA for the human NRF2 (Invitrogen, Zug, Switzerland) and for ATF3 (GeneGlobe, Qiagen, Basel, Switzerland) in parallel with corresponding scrambled siRNA controls and analyzed 12 and 72 hours, respectively, after transfection.

UV irradiation

The UV irradiation experiments were performed as described previously (Dziunycz *et al.*, 2010).

Reverse transcription and quantitative real-time PCR

The reverse transcription and quantitative real-time PCR was performed as described previously (Kosmidis *et al.*, 2010). The primer sequence is listed in the Supplementary Data online.

Western blot and antibodies

Western blotting was performed as described previously (Restivo *et al.*, 2011). The following antibodies were used: actin (sc-1616), NRF2 (sc-365949), ATF3 (sc-188), and NFATc1 (sc-7294; Santa Cruz, Heidelberg, Germany).

Chromatin immunoprecipitation assay

In all, 80% confluent primary human keratinocytes were left untreated or were irradiated with UVA at 5 J cm^{-2}. One hour later, cells were cross-linked with 37% formaldehyde in a final concentration of 1% for 10 minutes at room temperature followed by the addition of glycine (final concentration 125 mM). After cross-linking, cells were washed twice with 10 ml phosphate-buffered saline with protease inhibitor. Cell pellets were processed for chromatin immunoprecipitation assays using the Magna ChIP A/G Kit (Millipore, Zug, Switzerland) following the manufacturer's protocol. The anti-NRF2 antibody (Santa Cruz, sc365949) in parallel with affinity-purified nonimmune IgGs was used. Primers used for real-time PCR are listed in the Supplementary Data online.

Flow cytometry analysis of ROS formation

ROS formation was measured by flow cytometry. HKCs were cultured up to 70% of confluence. Before UVA exposure they were incubated for 20 minutes with CM-H2DCFDA dye (Molecular Probes, Eugene, OR). Cells were then irradiated with UVA at 0.1 J cm^{-2}, after which they were immediately trypsinized and analyzed. Measurements were performed on a FACSCanto device (BD Biosciences, Allschwil, Switzerland) as described (O'Donovan *et al.*, 2005). Data were analyzed with FlowJo software (Ashland, OR).

Statistics

All the experiments were performed at least three times. The graphs represent the compilation of the results. For western blot analysis, representative results have been chosen. All statistical evaluations were carried out using GraphPad Prism 5.0 (GraphPad, La Jolla, CA). The analyses were two-tailed Student's *t*-test or analysis of variance. All real-time reverse transcriptase-PCR samples were tested in triplicates, and error bars represent one standard deviation. *P* values of <0.05 were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Dantal J, Hourmant M, Cantarovich D *et al.* (1998) Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporin regimens. *Lancet* 351:623–8

de Gruijl FR (2002) Photocarcinogenesis: UVA vs. UVB radiation. *Skin Pharmacol Appl Skin Physiol* 15:316–20

Dotto GP (2011) Calcineurin signaling as a negative determinant of keratinocyte cancer stem cell potential and carcinogenesis. *Cancer Res* 71:2029–33

Dziunycz P, Iotzova-Weiss G, Eloranta JJ *et al.* (2010) Squamous cell carcinoma of the skin shows a distinct microRNA profile modulated by UV radiation. *J Invest Dermatol* 130:2686–9

Euvrard S, Morelon E, Rostaing L *et al.* (2012) Sirolimus and secondary skin-cancer prevention in kidney transplantation. *N Engl J Med* 367:329–39

Gruber F, Mayer H, Lengauer B *et al.* (2010) NF-E2-related factor 2 regulates the stress response to UVA-1-oxidized phospholipids in skin cells. *FASEB J* 24:39–48

Hoetzenecker W, Echtenacher B, Guenova E *et al.* (2012) ROS-induced ATF3 causes susceptibility to secondary infections during sepsis-associated immunosuppression. *Nat Med* 18:128–34

Hofbauer G (2010) [Immunosuppressive therapy after transplantation. Dermatologic relevance and pathomechanisms]. *Hautarzt* 61:214–9

Hofbauer GF, Attard NR, Harwood CA *et al.* (2012) Reversal of UVA skin photosensitivity and DNA damage in kidney transplant recipients by replacing azathioprine. *Am J Transplant* 12:218–25

Hofbauer GF, Bouwes Bavinck JN, Euvrard S (2010) Organ transplantation and skin cancer: basic problems and new perspectives. *Exp Dermatol* 19:473–82

IARC (2012) Agents Classified by the IARC Monographs, Volumes 1–105. http://monographs.iarc.fr/ENG/Classification/ClassificationsGroupOrder.pdf. Accessed on 21 October 2012

Jaramillo MC, Zhang DD (2013) The emerging role of the Nrf2-Keap1 signaling pathway in cancer. *Genes Dev* 27:2179–91

Kempf W, Mertz KD, Kanitakis J *et al.* (2012) Critical skin cancer in organ transplant recipients–a dermatopathological view. *Curr Probl Dermatol* 43:18–35

Khodagholi F, Tusi SK (2011) Stabilization of Nrf2 by tBHQ prevents LPS-induced apoptosis in differentiated PC12 cells. *Mol Cell Biochem* 354:97–112

Kolev V, Mandinova A, Guinea-Viniegra J *et al.* (2008) EGFR signalling as a negative regulator of Notch1 gene transcription and function in proliferating keratinocytes and cancer. *Nat Cell Biol* 10:902–11

Kosmidis M, Dziunycz P, Suarez-Farinas M *et al.* (2010) Immunosuppression affects CD4+ mRNA expression and induces Th2 dominance in the microenvironment of cutaneous squamous cell carcinoma in organ transplant recipients. *J Immunother* 33:538–46

Lindelof B, Dal H, Wolk K *et al.* (2005) Cutaneous squamous cell carcinoma in organ transplant recipients: a study of the Swedish cohort with regard to tumor site. *Arch Dermatol* 141:447–51

Marrot L, Jones C, Perez P *et al.* (2008) The significance of Nrf2 pathway in (photo)-oxidative stress response in melanocytes and keratinocytes of the human epidermis. *Pigment Cell Melanoma Res* 21:79–88

Morath C, Mueller M, Goldschmidt H *et al.* (2004) Malignancy in renal transplantation. *J Am Soc Nephrol* 15:1582–8

O'Donovan P, Perrett CM, Zhang X *et al.* (2005) Azathioprine and UVA light generate mutagenic oxidative DNA damage. *Science* 309:1871–4

Restivo G, Nguyen BC, Dziunycz P *et al.* (2011) IRF6 is a mediator of Notch pro-differentiation and tumour suppressive function in keratinocytes. *EMBO J* 30:4571–85

Thompson MR, Xu D, Williams BR (2009) ATF3 transcription factor and its emerging roles in immunity and cancer. *J Mol Med (Berl)* 87:1053–60

Wolfgang CD, Chen BP, Martindale JL *et al.* (1997) Gadd153/Chop10, a potential target gene of the transcriptional repressor ATF3. *Mol Cell Biol* 17:6700–7

Wu X, Nguyen BC, Dziunycz P *et al.* (2010) Opposing roles for calcineurin and ATF3 in squamous skin cancer. *Nature* 465:368–72

Table S1. Putative ATF3 downstream targets selected by cDNA microarray analysis.

	Fold change	
	HKC AdATF3	HKC siATF3
KCNN4	1.61	-3.97
VNN1	1.94	-2.69
CYP1A1	-2.27	2.5
NEFL	-1.5	1.97

Normal human keratinocytes were infected with ATF3 overexpressing adenovirus (HKC AdATF3) or control empty virus. On the other hand cells were transfected with siRNA specific for ATF3 (HKC siATF3) or control scrambled RNA. After 48 hours cellular RNA was isolated and microarray analysis was performed as described previously (Wu *et al.*, 2010).

Table S2. Primers (human genes) used in the real time RT-PCR experiments

GENE	FORWARD	REVERSE
ATF3	ACGGAGTGCCTGCAGAAAG	TCTCGTTCTTGAGCTCCTCA
ATF2	GGCACAAACTGCACAGCCCA	GGAAATGGGCCTGGTACAGGG
ATF4	CCAGTCGGGTTTGGGGGCTG	GGAGGAGCCCGCCTTAGCCT
c-Fos	GCCTGTCAACGCGCAGGACT	GCTCTGGTCTGCGATGGGGC
c-Jun	GGGAGTGGAGGTGCGCGGAGTCAGG	GAAACACCAGCCCGGGAGCCACAGG
JunB	AGATGAACCACGTGACACCC	GGAGTAGCTGCTGAGGTTGG
JunD	GCGCCTGGAAGAGAAAGTGA	GTTGACGTGGCTGAGGACTT
Fra-1	ATGGTACAGCCTCATTTCCTGG	CGGGCTGATCTGTTCACAAGG
Fra-2	GCATCTCCCTCCGAATCCTG	GGGTTACAGAGCCAGCAGAG
FosB	CCT TCC CCG TTG TTA ACC CT	CGA GTT CAG GGG ATC GGA
VNN1	GAACCCAGTATGTCTTTCCT	CATACAACCTCCCAAACAGA
KCNN4	TGGGCGGGGATCTGGTGCTT	AGGTAGAGCGCCCACGAGCA
GADD153	GCACCTCCCAGAGCCCTCACT	ATGCGCTGCTTTCCAGCCCG
36B4	GCAATGTTGCCAGTGTCTGT	GCCTTGACCTTTTCAGCAAG
ATF3 promoter region -3.5k bp	TACCACTACTGAGGGCTGACTGG	CATTGTCAGGAAACAGGGGC
ATF3 promoter region -2.9k bp	GTGCCACATCCTCAGAGAAATG	CACAGTAGGCACCAAAGTGTGG

Figure S1

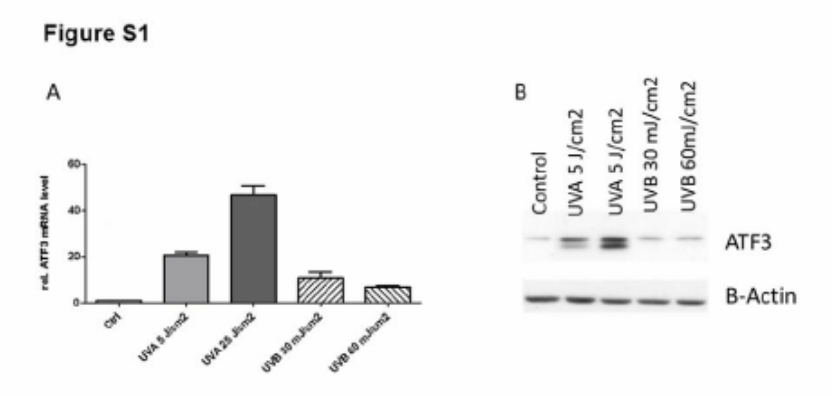


Figure S1. UVA but not UVB is a potent inducer of ATF3 expression. Cultures normal human keratinocytes at 80% of confluence were exposed to different doses of UVA (5 J/cm2 and 25 J/cm2) or UVB (30 mJ/cm2 and 60 mJ/cm2) radiation. At the time of the irradiation cells were kept at constant room temperature. After the irradiation cells were incubated for further 6 hours. After this time-frame cells were homogenized for RNA and protein extraction. ATF3 expression was next measured at both (A) mRNA and (B) protein levels.

Figure S2

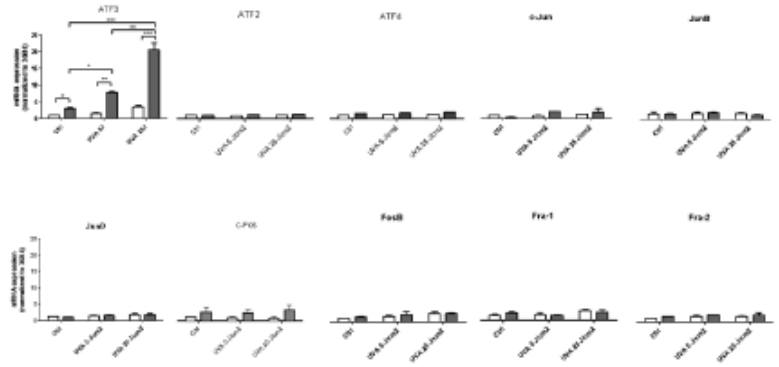


Figure S2. Within the enlarged AP-1 family members ATF3 expression was selectively and strongly up-regulated upon combined CsA and UVA treatment. Cultures human keratinocytes were treated with 3.3 uM CsA for 6 hours and then exposed to UVA at 5 J/cm2 or 25J/cm2. After 6 hours from the end of irradiation, mRNA was extracted and the expression of ATF3, ATF2, ATF4, c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2 was measured by quantitative real-time PCR. The open bars represent the gene expression in vehicle treated cells, the closed bars represent the gene expression in CsA treated cells. The results are represented as mean +/- SD.

Figure S3

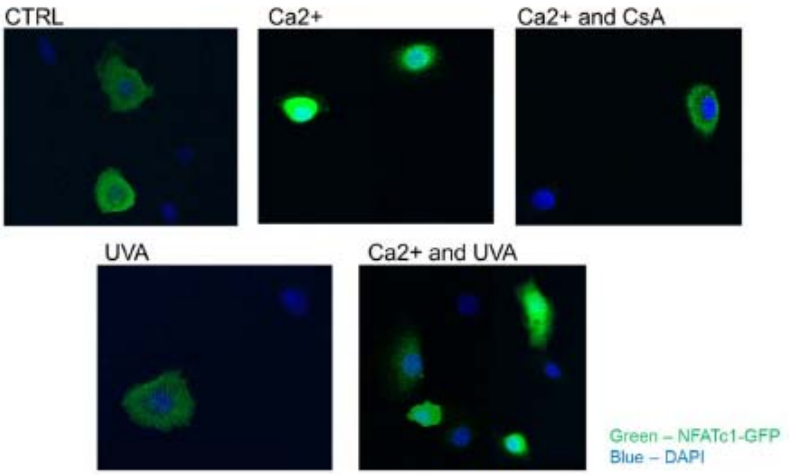


Figure S3. The UVAirradiation does not influence NFATc1 nuclear translocation. Primary human keratinocytes were transfected with a plasmid overexpressing NFATc1 fused with GFP. Three days after transfection, the influence of UVA irradiation on NFATc1 nuclear translocation was measured. Treatment with Ca2+ strongly induced the translocation of NFATc1 to the nucleus. This could be inhibited if the cells were pretreated with CsA. UVA treatment alone had no effect on the nuclear shift of NFATc1. UVA irradiation also did not inhibit the Ca2+ induced activation of NFATc1.

Reference

Wu X, Nguyen BC, Dziunycz P, Chang S, Brooks Y, Lefort K, *et al.* (2010) Opposing roles for calcineurin and ATF3 in squamous skin cancer. *Nature* 465:368-72.

VI. Discussion

The incidence of actinic keratosis (AK) and squamous cell carcinoma (SCC) of the skin has been rising during the last decades, making SCC the second most common human skin cancer (Hollestein et al., 2012). The lesions are caused by chronic exposure to sunlight, more specific by UV irradiation. Therefore, the protection from sun and UV by using sunscreen, avoiding midday sun or wearing long-sleeve cloths is highly recommended (Naylor et al., 1995; Thompson et al., 1993). In spite of increased awareness of the need for sun protection, the time lag between sun damage and onset of skin cancer formation still leads to increasing numbers of patients with AK and SCC. In addition, organ transplant recipients (OTR) carry a dramatically increased risk to develop AK and SCC due to the intake of immunosuppressive drugs (Dantal et al., 1998; Stockfleth et al., 2002). A recent report revealed that SCCs can develop directly from KIN I lesions in the basal keratinocyte layer and do not necessarily develop through KIN II and KIN III lesions (Fernandez-Figueras et al., 2014). New estimates on the number of deaths arising from invasive SCC in the United States show that about 2.1% of patients died from SCC (Karia et al., 2013). Clinically, AK is treated routinely under the assumption that by treatment at this stage, invasive SCC will be avoided. While there are several options to treat AK, invasive SCC usually gets excised. AK can be treated using cryotherapy, photodynamic therapy, radiotherapy or topical treatments (Euroderm, 2011).

In former times, people were using natural products to treat various types of diseases, among these products, the sap of the plant *E. peplus*. A survey in the Australian population revealed that this treatment actually was effective against skin cancer (Green and Beardmore, 1988), leading to the investigation of the constituents of the sap and eventually to the discovery of the active compound ingenol mebutate (IM) (Aylward, 2001). Both *in vitro* and *in vivo* studies showed the anti-cancer effectiveness of IM (Benhadji et al., 2008; Cozzi et al., 2013; Cozzi et al., 2012; Ogbourne et al., 2004). Meanwhile the drug has been registered and is routinely used in the treatment of AK (Lebwohl et al., 2012). However, the molecular mechanism of action of IM in keratinocytes is still unknown.

The main aim of the current thesis was to investigate the mechanism of action of IM in keratinocytes and SCC cells. By treating healthy and cancerous keratinocytes with varying IM concentrations for several time periods we could confirm previous reported anti-cancer activity of IM but also found healthy keratinocytes to be affected. However, healthy fibroblasts appeared to be resistant. Clinical studies show a rapid necrosis of treated lesions. Treatment of cell cultures with clinical IM concentrations resulted in mitochondrial disruption and direct cytotoxicity. Surprisingly, we found a biphasic effect of IM in keratinocytes, but not in other cell types tested, hinting at two different mechanisms of action. Referring to the previously mentioned study we suggest direct cytotoxicity as effect on cell viability at the highest concentration used. The intermediate IM concentration, however, may trigger intracellular pathways leading to reduced cell viability. As the concentration of IM when applied on the skin is decreasing with depth and is very low in the dermis, it is likely that basal keratinocytes react different than keratinocytes in the upper part of the epidermis.

Pathway analysis following gene expression profiling confirmed a number of affected cellular processes; among them the reduction of cell cycle associated genes, explaining the decreased proliferation capacity. Gene expression analysis further revealed an overlap of differentially expressed genes between treated keratinocytes and SCC cells with some genes differentially regulated. Since we found no differences in cell viability between keratinocytes and SCC cells, we focused on investigating the overlapping genes and with that on the overall IM effect. However, in preliminary experiments we had analyzed differentiation markers after IM treatment and found them induced in keratinocytes while they remained constant in SCC cells (data not shown). To further investigate this effect, more experiments would be needed.

In concordance with previous studies on other cell types (Benhadji et al., 2008; Cozzi et al., 2006) our results indicate an involvement of protein kinase C δ (PKCδ) in the mechanism of action of IM. PKCδ is a key mediator of several cellular processes including differentiation and apoptosis (Bowles et al., 2007; Hernandez-Maqueda et al., 2013; Papp et al., 2004; Zhang et al., 2014) and was shown to interact with ingenol mebutate (Kedei et al., 2004). Furthermore, we found ERK1/2 to be phosphorylated in a PKCδ-dependent manner after IM treatment. While studies in other cancer cells

suggested an involvement of the MAPK pathways JNK and p38 as well (Benhadji et al., 2008; Serova et al., 2008), we could exclude such an involvement in our setting. Commonly, the MEK/ERK pathway is considered to promote cell proliferation with numerous reports supporting this assumption. High activation of this pathway for example is associated with poor prognosis in colorectal cancer (Schmitz et al., 2007). Furthermore, it is well known that mutations in *RAS* or *RAF* cause constitutive activation of the MEK/ERK pathway in melanoma (Davies et al., 2002; Padua et al., 1985). However, we found both MEK and ERK activation to be essential for the performance of IM. This can be explained by the fact that ERK activation was actually also shown to suppress tumor growth and inversely correlates with the Gleason score, a grading system for prostate cancer (Deschenes-Simard et al., 2013). Furthermore, ERK activation is associated with good prognostic outcome in clear cell renal cell carcinoma (Lee et al., 2009), endometrial cancer (Mizumoto et al., 2007), prostate cancer and breast cancer (Malik et al., 2002; Svensson et al., 2005). In line with these findings, we see a lower phospho-ERK/ERK ratio in SCC cells compared to keratinocytes, and even a lower ratio in the SCC cell line (data not shown). Our preliminary results using an ERK inhibitor on primary keratinocytes revealed no growth inhibition at concentrations up to 1 µM (data not shown), while the same ERK inhibitor was shown to strongly decrease proliferation of melanoma cells (Morris et al., 2013). All that indicates that ERK phosphorylation may play a tumor-suppressing role in SCC and could be a candidate for new therapeutic approaches.

Our gene expression analysis provided a great number of differentially expressed genes after treatment overlapping between keratinocytes and SCC cells. Two of them, IL1R2 and IL13RA2, were selected for further analysis. The fact that both genes are commonly known as decoy receptors, not exerting any signaling function, made them interesting candidates. Keratinocytes have a certain inflammatory activity as they are able to produce and secrete a variety of cytokines (Bos and Kapsenberg, 1993). Furthermore, for the mechanism of action of IM, a neutrophil infiltration triggered by necrotic keratinocytes was reported (Rosen et al., 2012). However, our interest focused on the impact of IM on keratinocytes, apart from the immune system and we were wondering whether the encountered decoy receptors can directly influence the viability of the cells.

While IL1R2 was found to be upregulated in tumors like prostate cancer or adenocarcinoma, it has a low expression level in endometriosis and endometroid ovarian cancer (Garlanda et al., 2013). IL1R2 has a high binding affinity to IL1β and thereby prevents its binding to IL1R1, which has a signaling capacity (Boraschi and Tagliabue, 2013; Colotta et al., 1994). In a study on oral SCC it was demonstrated that IL1β promotes tumor growth by increasing the proliferation of malignant keratinocytes (Lee et al., 2015). It is possible that IL1R2, which we found to be upregulated after IM treatment, has the function to bind IL1β to prevent its effect on cell proliferation. Supporting this hypothesis, Khoufache et al. showed that soluble IL1R2 can inhibit endometrial tissue growth and decreases the expression of anti-apoptotic proteins (Khoufache et al., 2012). In our knockdown experiments we showed that the absence of IL1R2 increased the viability of IM-treated cells, indicating a regulatory function of this receptor. However, to further investigate the role of IL1R2 and its mechanism in regulating cell proliferation additional experiments will be needed and can be addressed in a separate project.

IL13AR2 was found to be overexpressed in glioma (Bernard et al., 2001). However, it has been shown to elicit inhibitory effects on breast cancer and pancreatic cancer cells. Here, cancer cells overexpressing the receptor were injected into nude mice and reduced or even prevented tumor growth compared to control cancer cells (Kawakami et al., 2001a). Consistently, we found IL13RA2 to be upregulated after IM treatment and the absence of this receptor improves the viability of IM-treated cells. Furthermore, IL13, the ligand of IL13RA2, was found to induce proliferation of human bronchial epithelial cells (Booth et al., 2007). Hence, IM-induced IL13RA2 may serve as a trap for IL13 to prevent it from stimulating cell proliferation.

While PKC inhibition could fully restore cell viability of the IM-treated SCC cell line, inhibition of ERK and MEK only partially fulfilled that task. Moreover, knockdown of IL1R2 and IL13RA2 only rescued the viability of IM-treated cells to a certain extent. These findings suggest that more processes then investigated in our study, downstream of PKC, contribute to the IM effect in keratinocytes and need to be further investigated.

Indicating a role of IL1R2 and IL13RA2 in the regulation of proliferation, we assume that certain other immune receptors on keratinocytes do not only play a role in the immune system, but also regulate cellular processes. Thus, we found RAGE, a receptor of the immunoglobulin superfamily, to be involved in signaling processes that led to increased keratinocyte proliferation. The capacity of the RAGE ligand S100A8/A9 to promote cell proliferation was previously reported for breast cancer cells (Ghavami et al., 2008). We could confirm this effect in keratinocytes and furthermore showed the involvement of RAGE in the regulation of proliferation. Several reports suggest a tumor-promoting role of RAGE in different types of cancer (Gebhardt et al., 2008; Xu et al., 2013; Zhao et al., 2014), matching our results on keratinocyte proliferation. These results indicate that RAGE could be a suitable target for cancer therapy in general and SCC in particular.

Furthermore, we suggest TLR4 to be a negative regulator for keratinocyte proliferation. The role of TLR4 in tumor development is controversially discussed as some reports show TLR4 expression to be beneficial for the tumor (Wang et al., 2010; Yang et al., 2010), but others reveal an association with tumor suppression (Gatti et al., 2009; Yusuf et al., 2008). This can be explained by the fact that the same protein can have opposing functions depending on the type of cell. A prominent example is Notch1, which is a tumor promoter in various cancer types like breast cancer, leukemia or lung cancer (Capaccione and Pine, 2013). In keratinocytes, Notch has an opposing role by promoting keratinocyte differentiation (Dotto, 2008).
Our results contribute to shed more light on the role of TLR4 in SCC development. Besides *in vitro* experiments, our mouse studies revealed a positive impact of TLR4 on tumor growth suppression, while TLR4-deficient keratinocytes gave rise to tumor formation. Whereas two contradictory reports show TLR4 knock-out mice to be either resistant or more susceptible to chemically-induced skin carcinogenesis, no data are available on cancer development after UV exposure on these mice. Such a study would help to better understand the role of SCC development as SCC in humans is mainly caused by UV. Moreover, TLR4 plays an important role in recognizing bacterial infections, therefore a mouse model with conditional knock-out in keratinocytes only, without affecting other cell types would contribute to the understanding of the function of TLR4 in SCC development. It remains to be elucidated whether TLR4 expression can

be regulated selectively in keratinocytes to treat SCC as its expression in other cell types is associated with tumor development (Mittal et al., 2010).

Another aim of the current PhD thesis was to investigate the role of CD32 in the context of keratinocyte proliferation and differentiation. To date, it is only known that CD32 is expressed on keratinocytes (Cauza et al., 2002) and is upregulated upon sulfur mustard treatment (Cowan et al., 1998), which causes keratinocyte differentiation (Popp et al., 2011). Our results indicate for the first time a link between CD32 and keratinocyte proliferation and differentiation. While blocking of CD32 resulted in increased proliferation, overexpression of the receptor resulted in a proliferation decrease. Consistently, CD32 was higher expressed in differentiated healthy cells and tissue. The higher expression of CD32 in poorly differentiated SCC compared to well differentiated SCC might be explained by a lack of receptor functionality. If the receptor is not activated or cannot bind to a ligand, it will not get internalized and thus accumulates on the cell surface.
Our data do not allow us to delineate a mechanism of action for CD32 in keratinocytes and SCC. It needs to be analyzed, whether CD32 increase is a consequence or a driver of differentiation. Here, knock-down experiments would clarify whether CD32 is essential for the process of cell differentiation. Furthermore, signaling pathways triggered by CD32 activation need to be investigated and the ligand that activates CD32 leading to decreased proliferation has to be found. CD32 commonly binds to the Fc portion of immunoglobulin G (IgG) (Ravetch and Kinet, 1991), however, there could be yet unknown binding partners of CD32 causing the observed effects.

OTR have a highly increased risk to develop SCC due to certain immunosuppressive drugs (Dantal et al., 1998; O'Donovan et al., 2005). Therefore it is recommended for them to avoid sunlight and protect themselves from UV exposure. Studies confirmed a benefit of such pronounced sun protection resulting in reduced SCC development (Ulrich et al., 2009). It remained to be elucidated whether CsA and UV act via similar pathways or cause SCC development independently. Our study on the transcription factor ATF3 which was previously shown to be highly expressed in SCC (Kim et al., 2011) and to promote tumorigenicity in mice (Wu et al., 2010), demonstrated the involvement of two different signaling pathways resulting in a potentiated ATF3

increase. While ATF3 was found to be increased by CsA treatment, nothing was known about the impact of UV, the main cause of SCC, on ATF3. Interestingly, we found UVA to increase ATF3 expression, while UVB exposure had no effect. This indicates an emerging role of UVA in the development of SCC. While UVB is causing DNA mutations, leading to tumor development, UVA induces ROS, leading to ATF3 induction. Unlike UVA, CsA induces ATF3 expression via the inhibition of the calcineurin/NFATc1 pathway. Thus, both CsA and UVA exposure result in ATF3-mediated p53 suppression, while UVB causes mutations in p53 among others. Furthermore, UV exposure potentiating the effect of CsA on ATF3 could explain the greatly increased incidence of SCC on sun-exposed surfaces of the skin in OTR.

Overall the current PhD thesis contributes to a better understanding of the mechanism of action of ingenol mebutate and helps to improve ingenol-based treatments. Currently, we are further investigating the phospho protein pattern in treated normal skin and SCC tissue to find new targets and more involved pathways. Furthermore, the thesis adds knowledge in the identification of new potential key mediators in the process of SCC development, suggesting them as candidates for new therapeutic approaches.

VII. References

Ackerman, A.B., and Mones, J.M. (2006). Solar (actinic) keratosis is squamous cell carcinoma. Br J Dermatol *155*, 9-22.

Adhikary, G., Chew, Y.C., Reece, E.A., and Eckert, R.L. (2010). PKC-delta and -eta, MEKK-1, MEK-6, MEK-3, and p38-delta are essential mediators of the response of normal human epidermal keratinocytes to differentiating agents. J Invest Dermatol *130*, 2017-2030.

Allison, R.R., and Moghissi, K. (2013). Photodynamic Therapy (PDT): PDT Mechanisms. Clin Endosc *46*, 24-29.

American Cancer Society (2014-08-11).

Arcoleo, J.P., and Weinstein, I.B. (1985). Activation of protein kinase C by tumor promoting phorbol esters, teleocidin and aplysiatoxin in the absence of added calcium. Carcinogenesis *6*, 213-217.

Aylward, J.H. (2001). Therapeutic agents - 1 International patent PCT/AU2001/000679.

Bacher, N., Zisman, Y., Berent, E., and Livneh, E. (1992). Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart. Mol Cell Biol *12*, 1404.

Barrado Solis, N., Moles Poveda, P., Lloret Ruiz, C., Pont Sanjuan, V., Velasco Pastor, M., Quecedo Estebanez, E., and Miquel Miquel, J. (2014). Ingenol mebutate gel treatment for actinic cheilitis: report of four cases. Dermatologic therapy.

Benhadji, K.A., Serova, M., Ghoul, A., Cvitkovic, E., Le Tourneau, C., Ogbourne, S.M., Lokiec, F., Calvo, F., Hammel, P., Faivre, S*., et al.* (2008). Antiproliferative activity of PEP005, a novel ingenol angelate that modulates PKC functions, alone and in combination with cytotoxic agents in human colon cancer cells. Br J Cancer *99*, 1808-1815.

Bernard, D., Gosselin, K., Monte, D., Vercamer, C., Bouali, F., Pourtier, A., Vandenbunder, B., and Abbadie, C. (2004). Involvement of Rel/nuclear factor-kappaB transcription factors in keratinocyte senescence. Cancer Res *64*, 472-481.

Bernard, J., Treton, D., Vermot-Desroches, C., Boden, C., Horellou, P., Angevin, E., Galanaud, P., Wijdenes, J., and Richard, Y. (2001). Expression of interleukin 13 receptor in glioma and renal cell carcinoma: IL13Ralpha2 as a decoy receptor for IL13. Laboratory investigation; a journal of technical methods and pathology *81*, 1223-1231.

Bierhaus, A., Humpert, P.M., Morcos, M., Wendt, T., Chavakis, T., Arnold, B., Stern, D.M., and Nawroth, P.P. (2005). Understanding RAGE, the receptor for advanced glycation end products. Journal of molecular medicine *83*, 876-886.

Birch-Johansen, F., Jensen, A., Mortensen, L., Olesen, A.B., and Kjaer, S.K. (2010). Trends in the incidence of nonmelanoma skin cancer in Denmark 1978-2007: Rapid incidence increase among young Danish women. Int J Cancer *127*, 2190-2198.

Booth, B.W., Sandifer, T., Martin, E.L., and Martin, L.D. (2007). IL-13-induced proliferation of airway epithelial cells: mediation by intracellular growth factor mobilization and ADAM17. Respir Res *8*, 51.

Boraschi, D., and Tagliabue, A. (2013). The interleukin-1 receptor family. Seminars in immunology *25*, 394-407.

Bos, J.D., and Kapsenberg, M.L. (1993). The skin immune system: progress in cutaneous biology. Immunology today *14*, 75-78.

Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N.E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol *106*, 761-771.

Bowles, D.K., Maddali, K.K., Dhulipala, V.C., and Korzick, D.H. (2007). PKCdelta mediates anti-proliferative, pro-apoptic effects of testosterone on coronary smooth muscle. American journal of physiology Cell physiology *293*, C805-813.

Bradham, C., and McClay, D.R. (2006). p38 MAPK in development and cancer. Cell Cycle *5*, 824-828.

Brash, D.E. (2006). Roles of the transcription factor p53 in keratinocyte carcinomas. Br J Dermatol *154 Suppl 1*, 8-10.

Brash, D.E., Rudolph, J.A., Simon, J.A., Lin, A., McKenna, G.J., Baden, H.P., Halperin, A.J., and Ponten, J. (1991). A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci U S A *88*, 10124-10128.

Capaccione, K.M., and Pine, S.R. (2013). The Notch signaling pathway as a mediator of tumor survival. Carcinogenesis *34*, 1420-1430.

Cassarino, D.e.a. (2012). Diagnostic Pathology - Neoplastic Dermatopathology, First edition edn (Amirsys).

Cauza, K., Grassauer, A., Hinterhuber, G., Horvat, R., Rappersberger, K., Wolff, K., and Foedinger, D. (2002). FcgammaRIII expression on cultured human keratinocytes and upregulation by interferon-gamma. J Invest Dermatol *119*, 1074-1079.

Challacombe, J.M., Suhrbier, A., Parsons, P.G., Jones, B., Hampson, P., Kavanagh, D., Rainger, G.E., Morris, M., Lord, J.M., Le, T.T.*, et al.* (2006). Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with ingenol-3-angelate. J Immunol *177*, 8123-8132.

Chen, W., Tabata, Y., Gibson, A.M., Daines, M.O., Warrier, M.R., Wills-Karp, M., and Hershey, G.K. (2008). Matrix metalloproteinase 8 contributes to solubilization of IL-13 receptor alpha2 in vivo. The Journal of allergy and clinical immunology *122*, 625-632.

Cohen, E.E., Lingen, M.W., Zhu, B., Zhu, H., Straza, M.W., Pierce, C., Martin, L.E., and Rosner, M.R. (2006). Protein kinase C zeta mediates epidermal growth factor-induced growth of head and neck tumor cells by regulating mitogen-activated protein kinase. Cancer Res *66*, 6296-6303.

Colotta, F., Dower, S.K., Sims, J.E., and Mantovani, A. (1994). The type II 'decoy' receptor: a novel regulatory pathway for interleukin 1. Immunology today *15*, 562-566.

Cosmic (2014). Catalogue of somatic mutations in cancer.

Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U., and Ullrich, A. (1986). Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science *233*, 859-866.

Cowan, F.M., Broomfield, C.A., and Smith, W.J. (1998). Sulfur mustard exposure enhances Fc receptor expression on human epidermal keratinocytes in cell culture: implications for toxicity and medical countermeasures. Cell Biol Toxicol *14*, 261-266.

Cozzi, S.J., Le, T.T., Ogbourne, S.M., James, C., and Suhrbier, A. (2013). Effective treatment of squamous cell carcinomas with ingenol mebutate gel in immunologically intact SKH1 mice. Arch Dermatol Res *305*, 79-83.

Cozzi, S.J., Ogbourne, S.M., James, C., Rebel, H.G., de Gruijl, F.R., Ferguson, B., Gardner, J., Lee, T.T., Larcher, T., and Suhrbier, A. (2012). Ingenol mebutate field-directed treatment of UVB-damaged skin reduces lesion formation and removes mutant p53 patches. J Invest Dermatol *132*, 1263-1271.

Cozzi, S.J., Parsons, P.G., Ogbourne, S.M., Pedley, J., and Boyle, G.M. (2006). Induction of senescence in diterpene ester-treated melanoma cells via protein kinase C-dependent hyperactivation of the mitogen-activated protein kinase pathway. Cancer Res *66*, 10083-10091.

D'Costa, A.M., Robinson, J.K., Maududi, T., Chaturvedi, V., Nickoloff, B.J., and Denning, M.F. (2006). The proapoptotic tumor suppressor protein kinase C-delta is lost in human squamous cell carcinomas. Oncogene *25*, 378-386.

Daines, M.O., Tabata, Y., Walker, B.A., Chen, W., Warrier, M.R., Basu, S., and Hershey, G.K. (2006). Level of expression of IL-13R alpha 2 impacts receptor distribution and IL-13 signaling. J Immunol *176*, 7495-7501.

Dantal, J., Hourmant, M., Cantarovich, D., Giral, M., Blancho, G., Dreno, B., and Soulillou, J.P. (1998). Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporin regimens. Lancet *351*, 623-628.

Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W.*, et al.* (2002). Mutations of the BRAF gene in human cancer. Nature *417*, 949-954.

Denning, M.F. (2004). Epidermal keratinocytes: regulation of multiple cell phenotypes by multiple protein kinase C isoforms. The international journal of biochemistry & cell biology *36*, 1141-1146.

Deschenes-Simard, X., Gaumont-Leclerc, M.F., Bourdeau, V., Lessard, F., Moiseeva, O., Forest, V., Igelmann, S., Mallette, F.A., Saba-El-Leil, M.K., Meloche, S.*, et al.* (2013). Tumor suppressor activity of the ERK/MAPK pathway by promoting selective protein degradation. Genes Dev *27*, 900-915.

Deschenes-Simard, X., Kottakis, F., Meloche, S., and Ferbeyre, G. (2014). ERKs in cancer: friends or foes? Cancer Res *74*, 412-419.

Deucher, A., Efimova, T., and Eckert, R.L. (2002). Calcium-dependent involucrin expression is inversely regulated by protein kinase C (PKC)alpha and PKCdelta. J Biol Chem *277*, 17032-17040.

Donaldson, D.D., Whitters, M.J., Fitz, L.J., Neben, T.Y., Finnerty, H., Henderson, S.L., O'Hara, R.M., Jr., Beier, D.R., Turner, K.J., Wood, C.R.*, et al.* (1998). The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. J Immunol *161*, 2317-2324.

Dotto, G.P. (2008). Notch tumor suppressor function. Oncogene *27*, 5115-5123.

Drosten, M., Lechuga, C.G., and Barbacid, M. (2013). Genetic analysis of Ras genes in epidermal development and tumorigenesis. Small GTPases *4*, 236-241.

Euroderm (2011). Guidelines for the Management of Actinic Keratoses - Update 2011.

European Medicines Agency (2012). Assessment report Picato.

Fecker, L.F., Stockfleth, E., Nindl, I., Ulrich, C., Forschner, T., and Eberle, J. (2007). The role of apoptosis in therapy and prophylaxis of epithelial tumours by nonsteroidal anti-inflammatory drugs (NSAIDs). Br J Dermatol *156 Suppl 3*, 25-33.

Fernandez-Figueras, M.T., Carrato, C., Saenz, X., Puig, L., Musulen, E., Ferrandiz, C., and Ariza, A. (2014). Actinic keratosis with atypical basal cells (AK I) is the most common lesion associated with invasive squamous cell carcinoma of the skin. J Eur Acad Dermatol Venereol.

Frost, C., Williams, G., and Green, A. (2000). High incidence and regression rates of solar keratoses in a queensland community. J Invest Dermatol *115*, 273-277.

Garlanda, C., Riva, F., Bonavita, E., and Mantovani, A. (2013). Negative regulatory receptors of the IL-1 family. Seminars in immunology *25*, 408-415.

Gatti, G., Quintar, A.A., Andreani, V., Nicola, J.P., Maldonado, C.A., Masini-Repiso, A.M., Rivero, V.E., and Maccioni, M. (2009). Expression of Toll-like receptor 4 in the prostate gland and its association with the severity of prostate cancer. The Prostate *69*, 1387-1397.

Gebhardt, C., Riehl, A., Durchdewald, M., Nemeth, J., Furstenberger, G., Muller-Decker, K., Enk, A., Arnold, B., Bierhaus, A., Nawroth, P.P.*, et al.* (2008). RAGE signaling sustains inflammation and promotes tumor development. The Journal of experimental medicine *205*, 275-285.

Ghavami, S., Rashedi, I., Dattilo, B.M., Eshraghi, M., Chazin, W.J., Hashemi, M., Wesselborg, S., Kerkhoff, C., and Los, M. (2008). S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. Journal of leukocyte biology *83*, 1484-1492.

Gioeli, D., Mandell, J.W., Petroni, G.R., Frierson, H.F., Jr., and Weber, M.J. (1999). Activation of mitogen-activated protein kinase associated with prostate cancer progression. Cancer Res *59*, 279-284.

Glogau, R.G. (2000). The risk of progression to invasive disease. J Am Acad Dermatol *42*, 23-24.

Goto, Y., Arigami, T., Kitago, M., Nguyen, S.L., Narita, N., Ferrone, S., Morton, D.L., Irie, R.F., and Hoon, D.S. (2008). Activation of Toll-like receptors 2, 3, and 4 on human melanoma cells induces inflammatory factors. Mol Cancer Ther *7*, 3642-3653.

Grachtchouk, M., Pero, J., Yang, S.H., Ermilov, A.N., Michael, L.E., Wang, A., Wilbert, D., Patel, R.M., Ferris, J., Diener, J.*, et al.* (2011). Basal cell carcinomas in mice arise from hair follicle stem cells and multiple epithelial progenitor populations. J Clin Invest *121*, 1768-1781.

Green, A.C., and Beardmore, G.L. (1988). Home treatment of skin cancer and solar keratoses. Australas J Dermatol *29*, 127-130.

Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res *54*, 4855-4878.

Halata, Z., Grim, M., and Bauman, K.I. (2003). Friedrich Sigmund Merkel and his "Merkel cell", morphology, development, and physiology: review and new results. Anat Rec A Discov Mol Cell Evol Biol *271*, 225-239.

Hall, P.A., McKee, P.H., Menage, H.D., Dover, R., and Lane, D.P. (1993). High levels of p53 protein in UV-irradiated normal human skin. Oncogene *8*, 203-207.

Hampson, P., Chahal, H., Khanim, F., Hayden, R., Mulder, A., Assi, L.K., Bunce, C.M., and Lord, J.M. (2005). PEP005, a selective small-molecule activator of protein kinase C, has potent antileukemic activity mediated via the delta isoform of PKC. Blood *106*, 1362-1368.

Hampson, P., Kavanagh, D., Smith, E., Wang, K., Lord, J.M., and Ed Rainger, G. (2008). The anti-tumor agent, ingenol-3-angelate (PEP005), promotes the recruitment of cytotoxic neutrophils by activation of vascular endothelial cells in a PKC-delta dependent manner. Cancer immunology, immunotherapy : CII *57*, 1241-1251.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Heidenreich, B., Rachakonda, P.S., Hemminki, K., and Kumar, R. (2014). TERT promoter mutations in cancer development. Curr Opin Genet Dev *24*, 30-37.

Hernandez-Maqueda, J.G., Luna-Ulloa, L.B., Santoyo-Ramos, P., Castaneda-Patlan, M.C., and Robles-Flores, M. (2013). Protein kinase C delta negatively modulates canonical Wnt pathway and cell proliferation in colon tumor cell lines. PLoS One *8*, e58540.

Hirobe, T., Furuya, R., Akiu, S., Ifuku, O., and Fukuda, M. (2002). Keratinocytes control the proliferation and differentiation of cultured epidermal melanocytes from ultraviolet radiation B-induced pigmented spots in the dorsal skin of hairless mice. Pigment Cell Res *15*, 391-399.

Hofbauer, G.F., Attard, N.R., Harwood, C.A., McGregor, J.M., Dziunycz, P., Iotzova-Weiss, G., Straub, G., Meyer, R., Kamenisch, Y., Berneburg, M.*, et al.* (2012). Reversal of UVA skin photosensitivity and DNA damage in kidney transplant recipients by replacing azathioprine. Am J Transplant *12*, 218-225.

Hollestein, L.M., de Vries, E., and Nijsten, T. (2012). Trends of cutaneous squamous cell carcinoma in the Netherlands: increased incidence rates, but stable relative survival and mortality 1989-2008. Eur J Cancer *48*, 2046-2053.

Hsu, Y.C., Pasolli, H.A., and Fuchs, E. (2011). Dynamics between stem cells, niche, and progeny in the hair follicle. Cell *144*, 92-105.

Hu, B., Castillo, E., Harewood, L., Ostano, P., Reymond, A., Dummer, R., Raffoul, W., Hoetzenecker, W., Hofbauer, G.F., and Dotto, G.P. (2012). Multifocal epithelial tumors and field cancerization from loss of mesenchymal CSL signaling. Cell *149*, 1207-1220.

IEWF (International Environmental Weed Foundation).

Ikehata, H., and Ono, T. (2011). The mechanisms of UV mutagenesis. Journal of radiation research *52*, 115-125.

Javed, S., and Tyring, S.K. (2014). Treatment of molluscum contagiosum with ingenol mebutate. J Am Acad Dermatol *70*, e105.

Jerome-Morais, A., Rahn, H.R., Tibudan, S.S., and Denning, M.F. (2009). Role for protein kinase C-alpha in keratinocyte growth arrest. J Invest Dermatol *129*, 2365-2375.

Jonason, A.S., Kunala, S., Price, G.J., Restifo, R.J., Spinelli, H.M., Persing, J.A., Leffell, D.J., Tarone, R.E., and Brash, D.E. (1996). Frequent clones of p53-mutated keratinocytes in normal human skin. Proc Natl Acad Sci U S A *93*, 14025-14029.

Jorgensen, L., McKerrall, S.J., Kuttruff, C.A., Ungeheuer, F., Felding, J., and Baran, P.S. (2013). 14-step synthesis of (+)-ingenol from (+)-3-carene. Science *341*, 878-882.

Kalluri, R., and Zeisberg, M. (2006). Fibroblasts in cancer. Nat Rev Cancer *6*, 392-401.

Karia, P.S., Han, J., and Schmults, C.D. (2013). Cutaneous squamous cell carcinoma: estimated incidence of disease, nodal metastasis, and deaths from disease in the United States, 2012. J Am Acad Dermatol *68*, 957-966.

Kawakami, K., Kawakami, M., Snoy, P.J., Husain, S.R., and Puri, R.K. (2001a). In vivo overexpression of IL-13 receptor alpha2 chain inhibits tumorigenicity of human breast and pancreatic tumors in immunodeficient mice. The Journal of experimental medicine *194*, 1743-1754.

Kawakami, K., Taguchi, J., Murata, T., and Puri, R.K. (2001b). The interleukin-13 receptor alpha2 chain: an essential component for binding and internalization but not for

interleukin-13-induced signal transduction through the STAT6 pathway. Blood *97*, 2673-2679.

Kedei, N., Lundberg, D.J., Toth, A., Welburn, P., Garfield, S.H., and Blumberg, P.M. (2004). Characterization of the interaction of ingenol 3-angelate with protein kinase C. Cancer Res *64*, 3243-3255.

Khoufache, K., Bondza, P.K., Harir, N., Daris, M., Leboeuf, M., Mailloux, J., Lemyre, M., Foster, W., and Akoum, A. (2012). Soluble human IL-1 receptor type 2 inhibits ectopic endometrial tissue implantation and growth: identification of a novel potential target for endometriosis treatment. The American journal of pathology *181*, 1197-1205.

Kim, M.S., In, S.G., Park, O.J., Won, C.H., Lee, M.W., Choi, J.H., Kim, C.W., Kim, S.E., Moon, K.C., and Chang, S. (2011). Increased expression of activating transcription factor 3 is related to the biologic behavior of cutaneous squamous cell carcinomas. Hum Pathol *42*, 954-959.

Knippers, R. (2001). Molekulare Genetik, 8. überarbeitete Auflage edn (Thieme).

Koul, H.K., Pal, M., and Koul, S. (2013). Role of p38 MAP Kinase Signal Transduction in Solid Tumors. Genes & cancer *4*, 342-359.

Krawtchenko, N., Roewert-Huber, J., Ulrich, M., Mann, I., Sterry, W., and Stockfleth, E. (2007). A randomised study of topical 5% imiquimod vs. topical 5-fluorouracil vs. cryosurgery in immunocompetent patients with actinic keratoses: a comparison of clinical and histological outcomes including 1-year follow-up. Br J Dermatol *157 Suppl 2*, 34-40.

Kypriotou, M., Huber, M., and Hohl, D. (2012). The human epidermal differentiation complex: cornified envelope precursors, S100 proteins and the 'fused genes' family. Exp Dermatol *21*, 643-649.

LaPak, K.M., and Burd, C.E. (2014). The molecular balancing act of p16(INK4a) in cancer and aging. Molecular cancer research : MCR *12*, 167-183.

Lapouge, G., Youssef, K.K., Vokaer, B., Achouri, Y., Michaux, C., Sotiropoulou, P.A., and Blanpain, C. (2011). Identifying the cellular origin of squamous skin tumors. Proc Natl Acad Sci U S A *108*, 7431-7436.

Lebwohl, M., Dinehart, S., Whiting, D., Lee, P.K., Tawfik, N., Jorizzo, J., Lee, J.H., and Fox, T.L. (2004). Imiquimod 5% cream for the treatment of actinic keratosis: results from two phase III, randomized, double-blind, parallel group, vehicle-controlled trials. J Am Acad Dermatol *50*, 714-721.

Lebwohl, M., Swanson, N., Anderson, L.L., Melgaard, A., Xu, Z., and Berman, B. (2012). Ingenol mebutate gel for actinic keratosis. N Engl J Med *366*, 1010-1019.

Lee, C.H., Chang, J.S., Syu, S.H., Wong, T.S., Chan, J.Y., Tang, Y.C., Yang, Z.P., Yang, W.C., Chen, C.T., Lu, S.C.*, et al.* (2015). IL-1beta Promotes Malignant Transformation and Tumor Aggressiveness in Oral Cancer. J Cell Physiol *230*, 875-884.

Lee, C.H., Wu, S.B., Hong, C.H., Yu, H.S., and Wei, Y.H. (2013). Molecular Mechanisms of UV-Induced Apoptosis and Its Effects on Skin Residential Cells: The Implication in UV-Based Phototherapy. Int J Mol Sci *14*, 6414-6435.

Lee, H.J., Kim, D.I., Kang, G.H., Kwak, C., Ku, J.H., and Moon, K.C. (2009). Phosphorylation of ERK1/2 and prognosis of clear cell renal cell carcinoma. Urology *73*, 394-399.

Lefort, K., Mandinova, A., Ostano, P., Kolev, V., Calpini, V., Kolfschoten, I., Devgan, V., Lieb, J., Raffoul, W., Hohl, D*., et al.* (2007). Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases. Genes Dev *21*, 562-577.

LEOPharma (2013).

Lewis, D.A., and Spandau, D.F. (2007). UVB activation of NF-kappaB in normal human keratinocytes occurs via a unique mechanism. Arch Dermatol Res *299*, 93-101.

Li, L., Shukla, S., Lee, A., Garfield, S.H., Maloney, D.J., Ambudkar, S.V., and Yuspa, S.H. (2010). The skin cancer chemotherapeutic agent ingenol-3-angelate (PEP005) is a substrate for the epidermal multidrug transporter (ABCB1) and targets tumor vasculature. Cancer Res *70*, 4509-4519.

Liang, Y.J., Shyu, K.G., Wang, B.W., and Lai, L.P. (2006). C-reactive protein activates the nuclear factor-kappaB pathway and induces vascular cell adhesion molecule-1 expression through CD32 in human umbilical vein endothelial cells and aortic endothelial cells. J Mol Cell Cardiol *40*, 412-420.

Malbec, O., Fridman, W.H., and Daeron, M. (1999). Negative regulation of c-kit-mediated cell proliferation by Fc gamma RIIB. J Immunol *162*, 4424-4429.

Malik, S.N., Brattain, M., Ghosh, P.M., Troyer, D.A., Prihoda, T., Bedolla, R., and Kreisberg, J.I. (2002). Immunohistochemical demonstration of phospho-Akt in high Gleason grade prostate cancer. Clin Cancer Res *8*, 1168-1171.

Mansuy, M., Nikkels-Tassoudji, N., Arrese, J.E., Rorive, A., and Nikkels, A.F. (2014). Recurrent in situ melanoma successfully treated with ingenol mebutate. Dermatol Ther (Heidelb) *4*, 131-135.

McGrath, J.A., Eady, R.A.J., Pope, F.M. (2004). Rook´s textbook of Dermatology, Vol volume 1, Chapter 3, 7th Edition edn (Blackwell Science).

Miller, D.L., and Weinstock, M.A. (1994). Nonmelanoma skin cancer in the United States: incidence. J Am Acad Dermatol *30*, 774-778.

Mittal, D., Saccheri, F., Venereau, E., Pusterla, T., Bianchi, M.E., and Rescigno, M. (2010). TLR4-mediated skin carcinogenesis is dependent on immune and radioresistant cells. EMBO J *29*, 2242-2252.

Mizumoto, Y., Kyo, S., Mori, N., Sakaguchi, J., Ohno, S., Maida, Y., Hashimoto, M., Takakura, M., and Inoue, M. (2007). Activation of ERK1/2 occurs independently of KRAS or BRAF status in endometrial cancer and is associated with favorable prognosis. Cancer science *98*, 652-658.

Mizuno, K., Okamoto, H., and Horio, T. (2004). Ultraviolet B radiation suppresses endocytosis, subsequent maturation, and migration activity of langerhans cell-like dendritic cells. J Invest Dermatol *122*, 300-306.

Moriyama, M., Durham, A.D., Moriyama, H., Hasegawa, K., Nishikawa, S., Radtke, F., and Osawa, M. (2008). Multiple roles of Notch signaling in the regulation of epidermal development. Dev Cell *14*, 594-604.

Morris, E.J., Jha, S., Restaino, C.R., Dayananth, P., Zhu, H., Cooper, A., Carr, D., Deng, Y., Jin, W., Black, S.*, et al.* (2013). Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. Cancer Discov *3*, 742-750.

Morton, C.A., Szeimies, R.M., Sidoroff, A., and Braathen, L.R. (2013). European guidelines for topical photodynamic therapy part 1: treatment delivery and current indications - actinic keratoses, Bowen's disease, basal cell carcinoma. J Eur Acad Dermatol Venereol *27*, 536-544.

Motley, R., Kersey, P., and Lawrence, C. (2002). Multiprofessional guidelines for the management of the patient with primary cutaneous squamous cell carcinoma. Br J Dermatol *146*, 18-25.

Mroz, P., Szokalska, A., Wu, M.X., and Hamblin, M.R. (2010). Photodynamic therapy of tumors can lead to development of systemic antigen-specific immune response. PLoS One *5*, e15194.

National Cancer Institute.

National Cancer Institute (2014a). Melanoma, N.C. Institute, ed.

National Cancer Institute (2014b). SEER Training Modul - Layers of the skin.

National Cancer Institute (2014c). skin cancer, N.C. Institute, ed.

Naylor, M.F., Boyd, A., Smith, D.W., Cameron, G.S., Hubbard, D., and Neldner, K.H. (1995). High sun protection factor sunscreens in the suppression of actinic neoplasia. Arch Dermatol *131*, 170-175.

Neeper, M., Schmidt, A.M., Brett, J., Yan, S.D., Wang, F., Pan, Y.C., Elliston, K., Stern, D., and Shaw, A. (1992). Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. J Biol Chem *267*, 14998-15004.

Nestle, F.O., Di Meglio, P., Qin, J.Z., and Nickoloff, B.J. (2009). Skin immune sentinels in health and disease. Nature reviews Immunology *9*, 679-691.

Nicolas, M., Wolfer, A., Raj, K., Kummer, J.A., Mill, P., van Noort, M., Hui, C.C., Clevers, H., Dotto, G.P., and Radtke, F. (2003). Notch1 functions as a tumor suppressor in mouse skin. Nat Genet *33*, 416-421.

Nimmerjahn, F., and Ravetch, J.V. (2008). Fcgamma receptors as regulators of immune responses. Nature reviews Immunology *8*, 34-47.

Nithianandarajah-Jones, G.N., Wilm, B., Goldring, C.E., Muller, J., and Cross, M.J. (2012). ERK5: structure, regulation and function. Cellular signalling *24*, 2187-2196.

O'Donovan, P., Perrett, C.M., Zhang, X., Montaner, B., Xu, Y.Z., Harwood, C.A., McGregor, J.M., Walker, S.L., Hanaoka, F., and Karran, P. (2005). Azathioprine and UVA light generate mutagenic oxidative DNA damage. Science *309*, 1871-1874.

Oblak, A., and Jerala, R. (2011). Toll-like receptor 4 activation in cancer progression and therapy. Clinical & developmental immunology *2011*, 609579.

Ogbourne, S.M., Suhrbier, A., Jones, B., Cozzi, S.J., Boyle, G.M., Morris, M., McAlpine, D., Johns, J., Scott, T.M., Sutherland, K.P.*, et al.* (2004). Antitumor activity of 3-ingenyl angelate: plasma membrane and mitochondrial disruption and necrotic cell death. Cancer Res *64*, 2833-2839.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1987). Identification of three additional members of rat protein kinase C family: delta-, epsilon- and zeta-subspecies. FEBS Lett *226*, 125-128.

Osada, S., Mizuno, K., Saido, T.C., Suzuki, K., Kuroki, T., and Ohno, S. (1992). A new member of the protein kinase C family, nPKC theta, predominantly expressed in skeletal muscle. Mol Cell Biol *12*, 3930-3938.

Ozaki, T., and Nakagawara, A. (2011). p53: the attractive tumor suppressor in the cancer research field. Journal of biomedicine & biotechnology *2011*, 603925.

Padua, R.A., Barrass, N.C., and Currie, G.A. (1985). Activation of N-ras in a human melanoma cell line. Mol Cell Biol *5*, 582-585.

Papp, H., Czifra, G., Bodo, E., Lazar, J., Kovacs, I., Aleksza, M., Juhasz, I., Acs, P., Sipka, S., Kovacs, L.*, et al.* (2004). Opposite roles of protein kinase C isoforms in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes. Cell Mol Life Sci *61*, 1095-1105.

Park, J.S., Gamboni-Robertson, F., He, Q., Svetkauskaite, D., Kim, J.Y., Strassheim, D., Sohn, J.W., Yamada, S., Maruyama, I., Banerjee, A.*, et al.* (2006). High mobility group box 1 protein interacts with multiple Toll-like receptors. American journal of physiology Cell physiology *290*, C917-924.

Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D., and Ullrich, A. (1986). The complete primary structure of protein kinase C--the major phorbol ester receptor. Science *233*, 853-859.

Pirard, D., Vereecken, P., Melot, C., and Heenen, M. (2005). Three percent diclofenac in 2.5% hyaluronan gel in the treatment of actinic keratoses: a meta-analysis of the recent studies. Arch Dermatol Res *297*, 185-189.

Popp, T., Egea, V., Kehe, K., Steinritz, D., Schmidt, A., Jochum, M., and Ries, C. (2011). Sulfur mustard induces differentiation in human primary keratinocytes: opposite roles of p38 and ERK1/2 MAPK. Toxicology letters *204*, 43-51.

Pritchard, A.L., and Hayward, N.K. (2013). Molecular pathways: mitogen-activated protein kinase pathway mutations and drug resistance. Clin Cancer Res *19*, 2301-2309.

Proweller, A., Tu, L., Lepore, J.J., Cheng, L., Lu, M.M., Seykora, J., Millar, S.E., Pear, W.S., and Parmacek, M.S. (2006). Impaired notch signaling promotes de novo squamous cell carcinoma formation. Cancer Res *66*, 7438-7444.

Ramsay, J.R., Suhrbier, A., Aylward, J.H., Ogbourne, S., Cozzi, S.J., Poulsen, M.G., Baumann, K.C., Welburn, P., Redlich, G.L., and Parsons, P.G. (2011). The sap from Euphorbia peplus is effective against human nonmelanoma skin cancers. Br J Dermatol *164*, 633-636.

Rangarajan, A., Talora, C., Okuyama, R., Nicolas, M., Mammucari, C., Oh, H., Aster, J.C., Krishna, S., Metzger, D., Chambon, P.*, et al.* (2001). Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J *20*, 3427-3436.

Rassner, G. (2007). Dermatologie, 8. Auflage edn (Elsevier Urban&Fischer).

Ravetch, J.V., and Kinet, J.P. (1991). Fc receptors. Annual review of immunology *9*, 457-492.

Regad, T. (2013). Molecular and cellular pathogenesis of melanoma initiation and progression. Cell Mol Life Sci *70*, 4055-4065.

Rheinwald, J.G., and Beckett, M.A. (1981). Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. Cancer Res *41*, 1657-1663.

Rosen, R., Freeman, M., Zibert, J.R., Katsamas, J., Knudsen, K.M., Spelman, L. (2014). Ingenol mebutate 0.05% gel reduces cancer cells in squamous cell carcinoma in situ and shows marginal effect in seborrhoeic keratosis. In World Congress on Cancers of the Skin.

Rosen, R.H., Gupta, A.K., and Tyring, S.K. (2012). Dual mechanism of action of ingenol mebutate gel for topical treatment of actinic keratoses: rapid lesion necrosis followed by lesion-specific immune response. J Am Acad Dermatol *66*, 486-493.

Rothberg, S., Crounse, R.G., and Lee, J.L. (1961). Glycine-C-14-incorporation into the proteins of normal stratum corneum and the abnormal straum corneum of psoriasis. J Invest Dermatol *37*, 497-505.

Rubin, A.I., Chen, E.H., and Ratner, D. (2005). Basal-cell carcinoma. N Engl J Med *353*, 2262-2269.

Salmon, J.K., Armstrong, C.A., and Ansel, J.C. (1994). The skin as an immune organ. The Western journal of medicine *160*, 146-152.

Schirren, C.G., Rutten, A., Kaudewitz, P., Diaz, C., McClain, S., and Burgdorf, W.H. (1997). Trichoblastoma and basal cell carcinoma are neoplasms with follicular differentiation sharing the same profile of cytokeratin intermediate filaments. The American Journal of dermatopathology *19*, 341-350.

Schmitz, K.J., Wohlschlaeger, J., Alakus, H., Bohr, J., Stauder, M.A., Worm, K., Winde, G., Schmid, K.W., and Baba, H.A. (2007). Activation of extracellular regulated kinases (ERK1/2) but not AKT predicts poor prognosis in colorectal carcinoma and is associated with k-ras mutations. Virchows Archiv : an international journal of pathology *450*, 151-159.

Schon, M.P., and Schon, M. (2007). Imiquimod: mode of action. Br J Dermatol *157 Suppl 2*, 8-13.

Serova, M., Ghoul, A., Benhadji, K.A., Faivre, S., Le Tourneau, C., Cvitkovic, E., Lokiec, F., Lord, J., Ogbourne, S.M., Calvo, F.*, et al.* (2008). Effects of protein kinase C modulation by PEP005, a novel ingenol angelate, on mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling in cancer cells. Mol Cancer Ther *7*, 915-922.

Setlow, R.B., and Carrier, W.L. (1966). Pyrimidine dimers in ultraviolet-irradiated DNA's. J Mol Biol *17*, 237-254.

Shang, L., Daubeuf, B., Triantafilou, M., Olden, R., Depis, F., Raby, A.C., Herren, S., Dos Santos, A., Malinge, P., Dunn-Siegrist, I.*, et al.* (2014). Selective antibody intervention of Toll-like receptor 4 activation through Fc gamma receptor tethering. J Biol Chem *289*, 15309-15318.

Siller, G., Gebauer, K., Welburn, P., Katsamas, J., and Ogbourne, S.M. (2009). PEP005 (ingenol mebutate) gel, a novel agent for the treatment of actinic keratosis: results of a randomized, double-blind, vehicle-controlled, multicentre, phase IIa study. Australas J Dermatol *50*, 16-22.

Siller, G., Rosen, R., Freeman, M., Welburn, P., Katsamas, J., and Ogbourne, S.M. (2010). PEP005 (ingenol mebutate) gel for the topical treatment of superficial basal cell carcinoma: results of a randomized phase IIa trial. Australas J Dermatol *51*, 99-105.

Simpson, C.L., Patel, D.M., and Green, K.J. (2011). Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. Nat Rev Mol Cell Biol *12*, 565-580.

Song, P.I., Park, Y.M., Abraham, T., Harten, B., Zivony, A., Neparidze, N., Armstrong, C.A., and Ansel, J.C. (2002). Human keratinocytes express functional CD14 and toll-like receptor 4. J Invest Dermatol *119*, 424-432.

Sorrell, J.M., and Caplan, A.I. (2004). Fibroblast heterogeneity: more than skin deep. J Cell Sci *117*, 667-675.

Stahlhut, M., Bertelsen, M., Hoyer-Hansen, M., Svendsen, N., Eriksson, A.H., Lord, J.M., Scheel-Toellner, D., Young, S.P., and Zibert, J.R. (2012). Ingenol mebutate: induced cell death patterns in normal and cancer epithelial cells. J Drugs Dermatol *11*, 1181-1192.

Stockfleth, E., Ulrich, C., Meyer, T., and Christophers, E. (2002). Epithelial malignancies in organ transplant patients: clinical presentation and new methods of treatment. Recent Results Cancer Res *160*, 251-258.

Svensson, S., Jirstrom, K., Ryden, L., Roos, G., Emdin, S., Ostrowski, M.C., and Landberg, G. (2005). ERK phosphorylation is linked to VEGFR2 expression and Ets-2 phosphorylation

in breast cancer and is associated with tamoxifen treatment resistance and small tumours with good prognosis. Oncogene *24*, 4370-4379.

Szeimies, R.M., Stockfleth, E., Popp, G., Borrosch, F., Bruning, H., Dominicus, R., Mensing, H., Reinhold, U., Reich, K., Moor, A.C.*, et al.* (2010). Long-term follow-up of photodynamic therapy with a self-adhesive 5-aminolaevulinic acid patch: 12 months data. Br J Dermatol *162*, 410-414.

Takahashi, H., Asano, K., Manabe, A., Kinouchi, M., Ishida-Yamamoto, A., and Iizuka, H. (1998). The alpha and eta isoforms of protein kinase C stimulate transcription of human involucrin gene. J Invest Dermatol *110*, 218-223.

Thai, K.E., Fergin, P., Freeman, M., Vinciullo, C., Francis, D., Spelman, L., Murrell, D., Anderson, C., Weightman, W., Reid, C.*, et al.* (2004). A prospective study of the use of cryosurgery for the treatment of actinic keratoses. Int J Dermatol *43*, 687-692.

Thompson, S.C., Jolley, D., and Marks, R. (1993). Reduction of solar keratoses by regular sunscreen use. N Engl J Med *329*, 1147-1151.

Tiu, J., Li, H., Rassekh, C., van der Sloot, P., Kovach, R., and Zhang, P. (2006). Molecular basis of posttransplant squamous cell carcinoma: the potential role of cyclosporine a in carcinogenesis. Laryngoscope *116*, 762-769.

Tournier, C. (2013). The 2 Faces of JNK Signaling in Cancer. Genes & cancer *4*, 397-400.

Tschen, E.H., Wong, D.S., Pariser, D.M., Dunlap, F.E., Houlihan, A., and Ferdon, M.B. (2006). Photodynamic therapy using aminolaevulinic acid for patients with nonhyperkeratotic actinic keratoses of the face and scalp: phase IV multicentre clinical trial with 12-month follow up. Br J Dermatol *155*, 1262-1269.

Ueda, E., Ohno, S., Kuroki, T., Livneh, E., Yamada, K., Yamanishi, K., and Yasuno, H. (1996). The eta isoform of protein kinase C mediates transcriptional activation of the human transglutaminase 1 gene. J Biol Chem *271*, 9790-9794.

Ulrich, C., Jurgensen, J.S., Degen, A., Hackethal, M., Ulrich, M., Patel, M.J., Eberle, J., Terhorst, D., Sterry, W., and Stockfleth, E. (2009). Prevention of non-melanoma skin cancer in organ transplant patients by regular use of a sunscreen: a 24 months, prospective, case-control study. Br J Dermatol *161 Suppl 3*, 78-84.

Verma, A.K., Wheeler, D.L., Aziz, M.H., and Manoharan, H. (2006). Protein kinase Cepsilon and development of squamous cell carcinoma, the nonmelanoma human skin cancer. Molecular carcinogenesis *45*, 381-388.

Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M.A., Nacken, W., Foell, D., van der Poll, T., Sorg, C.*, et al.* (2007). Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nature medicine *13*, 1042-1049.

Wang, E.L., Qian, Z.R., Nakasono, M., Tanahashi, T., Yoshimoto, K., Bando, Y., Kudo, E., Shimada, M., and Sano, T. (2010). High expression of Toll-like receptor 4/myeloid differentiation factor 88 signals correlates with poor prognosis in colorectal cancer. Br J Cancer *102*, 908-915.

Weinberg, A.S., Ogle, C.A., and Shim, E.K. (2007). Metastatic cutaneous squamous cell carcinoma: an update. Dermatol Surg *33*, 885-899.

Wheeler, D.L., Li, Y., and Verma, A.K. (2005). Protein kinase C epsilon signals ultraviolet light-induced cutaneous damage and development of squamous cell carcinoma possibly through Induction of specific cytokines in a paracrine mechanism. Photochem Photobiol *81*, 9-18.

Wheeler, D.L., Ness, K.J., Oberley, T.D., and Verma, A.K. (2003). Protein kinase Cepsilon is linked to 12-O-tetradecanoylphorbol-13-acetate-induced tumor necrosis factor-alpha ectodomain shedding and the development of metastatic squamous cell carcinoma in protein kinase Cepsilon transgenic mice. Cancer Res *63*, 6547-6555.

White, A.C., Tran, K., Khuu, J., Dang, C., Cui, Y., Binder, S.W., and Lowry, W.E. (2011). Defining the origins of Ras/p53-mediated squamous cell carcinoma. Proc Natl Acad Sci U S A *108*, 7425-7430.

Witkiewicz, A.K., Knudsen, K.E., Dicker, A.P., and Knudsen, E.S. (2011). The meaning of p16(ink4a) expression in tumors: functional significance, clinical associations and future developments. Cell Cycle *10*, 2497-2503.

Wu, A.H., and Low, W.C. (2003). Molecular cloning and identification of the human interleukin 13 alpha 2 receptor (IL-13Ra2) promoter. Neuro-oncology *5*, 179-187.

Wu, X., Nguyen, B.C., Dziunycz, P., Chang, S., Brooks, Y., Lefort, K., Hofbauer, G.F., and Dotto, G.P. (2010). Opposing roles for calcineurin and ATF3 in squamous skin cancer. Nature *465*, 368-372.

Xu, X.C., Abuduhadeer, X., Zhang, W.B., Li, T., Gao, H., and Wang, Y.H. (2013). Knockdown of RAGE inhibits growth and invasion of gastric cancer cells. European journal of histochemistry : EJH *57*, e36.

Yang, H., Zhou, H., Feng, P., Zhou, X., Wen, H., Xie, X., Shen, H., and Zhu, X. (2010). Reduced expression of Toll-like receptor 4 inhibits human breast cancer cells proliferation and inflammatory cytokines secretion. Journal of experimental & clinical cancer research : CR *29*, 92.

Yang, L.C., Ng, D.C., and Bikle, D.D. (2003). Role of protein kinase C alpha in calcium induced keratinocyte differentiation: defective regulation in squamous cell carcinoma. J Cell Physiol *195*, 249-259.

Yusuf, N., Nasti, T.H., Long, J.A., Naseemuddin, M., Lucas, A.P., Xu, H., and Elmets, C.A. (2008). Protective role of Toll-like receptor 4 during the initiation stage of cutaneous chemical carcinogenesis. Cancer Res *68*, 615-622.

Zhang, H., Okamoto, M., Panzhinskiy, E., Zawada, W.M., and Das, M. (2014). PKCdelta/midkine pathway drives hypoxia-induced proliferation and differentiation of human lung epithelial cells. American journal of physiology Cell physiology *306*, C648-658.

Zhang, J.G., Hilton, D.J., Willson, T.A., McFarlane, C., Roberts, B.A., Moritz, R.L., Simpson, R.J., Alexander, W.S., Metcalf, D., and Nicola, N.A. (1997). Identification, purification, and

characterization of a soluble interleukin (IL)-13-binding protein. Evidence that it is distinct from the cloned Il-13 receptor and Il-4 receptor alpha-chains. J Biol Chem *272*, 9474-9480.

Zhao, C.B., Bao, J.M., Lu, Y.J., Zhao, T., Zhou, X.H., Zheng, D.Y., and Zhao, S.C. (2014). Co-expression of RAGE and HMGB1 is associated with cancer progression and poor patient outcome of prostate cancer. American journal of cancer research *4*, 369-377.

Ziegler, A., Jonason, A.S., Leffell, D.J., Simon, J.A., Sharma, H.W., Kimmelman, J., Remington, L., Jacks, T., and Brash, D.E. (1994). Sunburn and p53 in the onset of skin cancer. Nature *372*, 773-776.

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AEG	Advanced glycosylation end product
ATF3	Activating transcription factor 3
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2A
Cfos	FBJ murine osteosarcoma viral oncogene homolog
CLSM	Confocal laser scanning microscope
COX-2	Ccyclooxygenase-2
CsA	Cyclosporine A
DMEM	Dulbecco´s modified eagle medium
DNA	Desoxyribonucleic acid
ECM	Extracellular matrix
EGR1	Early growth response 1
ERK	Extracellular regulated MAP kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
FcγR	Fcγ receptor
h	Hour
HCl	Hydrochloric acid
HNSCC	Head and neck squamous cell carcinoma
HRAS	Harvey rat sarcoma viral oncogene homolog
IgG	Immunoglobulin G
IL	Interleukin
IL13RA2	Interleukin-13 receptor alpha 2
IL1R2	Interleukin-1 receptor 2
IM	Ingenol mebutate
JNK	Jun amino-terminal kinase
KIN	Keratinocyte intraepidermal neoplasia
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
min	Minutes

OTR	Organ transplant recipient
PBS	Phosphate-buffered saline
PKC	Protein kinase C
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
sec	Seconds
SPF	Sun protection factor
SPRY2	Sprouty 2
TBS	Tris-buffered saline
TERT	Telomerase reverse transcriptase
TLR	Toll-like receptor
TPA	12-O-Tetradecanoylphorbol-13-acetate
USZ	University Hospital Zurich
UV	Ultra-violet
WB	Western blot
WT	Wild-type
XP	Xeroderma pigmentosum

Acknowledgements

I would like to thank all colleagues, friends and family who supported me for the last four years. Especially I would like to thank:

Prof. Günther Hofbauer for being my supervisor and giving me the opportunity to work in his group on this very interesting project. Also for the possibility to meet collaboration partners and to get to know other researchers on international conferences.

Prof. Reinhard Dummer for taking on the role of being my doctor father, for his support during my thesis and his input.

Prof. Paolo Dotto for being the external member of my PhD committee, for his input on the project and the helpful discussions on our lab visits at Lausanne.

Prof. Onur Boyman for being part of my PhD committee and his input in the yearly meetings.

Prof. Ian Frew, who kindly took over the part of the faculty member of my PhD committee.

Prof. Mitchell Levesque for his input in the project and always making time for discussing with me.

Dr. Guergana Iotzova-Weiss, who became a good friend, for introducing me to the lab work in dermatology and for her help on the project.

The “Schlieren-People” of the dermatology department, the former F14 crew: Dr. Phil Cheng, Dr. Antonia Fettelschoss, Dr. Marieke Raaijmakers and Niki Kobert for the great working atmosphere and especially Dr. Deepa Mohanan, Dr. Franziska Zabel and Dr. Daniel Widmer for their friendship and support, from going for sports through to solving computer problems, during the ups and downs on the way to the PhD.

Former and current colleagues of our group: Johannes Neu, Qinxiu Liu, Dr. Piotr Dziunycz and Dr. Paola Atzei.

Our collaborators from LEO Pharma, especially Dr. Kresten Skak and Dr. John Zibert for initiating this interesting project and for all their input and discussion. It was a pleasure to work with you and I always felt very welcome in Copenhagen.

Colleagues from the Dermatology Department, especially Dr. Pål Johansen for his help with the mouse experiments, Ines Kleiber-Schaaf for her help with immunohistochemistry and the biobank technicians for taking care of the clinical samples since we moved our lab.

Finally I want to thank my parents for their support. Especially I want to thank Niels for his understanding, his encouragement and for being there for me in any situation.